RAPPORT SUR LE MEMOIRE INTITULE :

"REGULATION DE LA REPONSE IMMUNITAIRE PAR LE PARASITE TREMATODE *SCHISTOSOMA MANSONI*"

présenté par Monsieur Claude AURIAULT pour l'obtention du Doctorat ès-Sciences Naturelles.

Le sujet du travail présenté par M. Claude AURIAULT est particulièrement intéressant puisqu'il envisage les relations du parasite *Schistosoma mansoni* avec son hôte et les processus utilisés par le parasite pour résister aux mécanismes de défense humorale ou cellulaire qui vise à le détruire.

Dans un chapitre d'introduction bien documenté et clairement rédigé, les modalités de réponse provoquée par la schistosomiase expérimentale chez le rat sont tout d'abord rappelées.

Les effets effecteurs sont représentés essentiellement par des mécanismes de cytotoxité cellulaire. Ils mettent en oeuvre les macrophages activés par les anticorps de nature IgE et les éosoniphiles associés à des IgG2 et à des IgE. Ainsi donc, chez *Schistoma*, comme dans d'autres modèles de parsites, l'indoction de l'activité phagocytaire est liée à la présence d'anticorps anaphylactiques.

Face à ces réactions de défense de l'organisme, le parasite développe des mécanismes d'évasion et d'immunorégulation : modulation membranaire, acquisition d'antigènes d'hôte, accepteurs de surface des schistosomes pour les IgG et la β-2 microglobuline. Enfin les schistosomes adultes sont capables d'exercer une modulation de l'activité de divers type de cellules, notamment des mastocytes.

Les résultats personnels sont présentés sous la forme d'un assemblage de 8 articles principaux rédigés en langue anglaise et publiés dans des revues internationales. Chaque article est précédé d'un préambule très clairement rédigé qui pose le but et la finalité de la recherche entreprise et dégage les principales caractéristiques des résultats obtenus.

Cette méthode, facilite considérablement pour le lecteur l'approche des problèmes et permet de faire ressortir la filiation unissant les différentes publications. La seule réserve qui puisse être émise concerne la difficulté de lecture de certaines figures des publications originales imparfaitement reproduites par la photocopie.

Les résultats obtenus se regroupent en 2 axes de recherches (A). Le clivage des IgG et ses incidences sur la réponse immune (B) Le rôle des protéases parasitaires dans l'activation des éosinophiles.

A - CLIVAGE DES Ig G ET INCIDENCE SUR LA REPONSE IMMUNE.

La surface des schistosomules de *Schistosoma mansoni* possède des récepteurs pour le fragment Fc des IgG. Après liaison des IgG avec la membrane, les portions Fab sont clivées par de petits peptides libérés dans le milieu de culture par les schistosomules. Deux catégories d'activités protéinasiques ont été détectées dans les produits de sécrétion. L'une est une endoprotéase avec une activité "trypsin-like". Un pH 7 et une température de 45°C constituent les conditions optimales fonctionnelles. L'autre est une métalloaminopeptidase (optimum = pH : 7, t : 37°C).

Les peptides libérés après le clivage des Ig G par les protéases sécrétées par les schistosomales exercent une action inhibitrice sur l'activité des macrophages. On note en effet une inhibition de 50 à 70 % de la cytotoxité IgE - dépendante exercée par les macrophages contre les schistosomules lorsque les peptides précédemment cités sont ajoutés au milieu de culture.

Cet effet inhibiteur exercé par les peptides d'Ig G est non spécifique puisqu'il provoque également l'inhibition d'autres activités macrophagiques telles la libération de la β-glucuronidase, l'incorporation de glucosamine, la phagocytose des particules de latex.

Ce processus peut constituer pour le parasite un efficient mécanisme immunosuppresseur lui permettant d'échapper à la défense de l'hôte.

Les recherches ont ensuite portées sur la nature du facteur actif résultant du clivage des molécules d'Ig G. En utilisant notamment le test du relarguage de la β-glucuronidase, M. AURIAULT démontre qu'il s'agit du tripeptide (Threonyl-Lysyl-Proline) du second domaine constant de l'immunoglobuline G. Ce tripeptide inhibe également la migration des macrophages de rat ainsi que la génération d'ions superoxydes.

Les travaux relatifs à ce premier axe de recherches se terminent par l'examen du rôle des fragments Fc maintenus en place sur la membrane des schistosomules. L'auteur montre que ces derniers, restés à la surface après le clivage des Ig G par les protéases parasitaires, entraînent l'activation du complément par la voie classique comme en témoignent les consommations des facteurs C_1-C_2 et C_4. Ces résultats permettent à l'auteur de formuler l'hypothèse que ce mécanisme, en provoquant une consommation locale du complément autour de la schistosomule pourrait constituer un des mécanismes de sa survie à l'intérieur de l'hôte.

B - ROLE DES PROTEASES PARASITAIRES DANS L'ACTIVATION DES EOSINOPHILES.

Si les peptides d'Ig G sont sans action sur la fonction effectrice des éosinophiles, par contre les agents de sécrétion des larves exercent un effet stimulant sur leur activité comme en témoigne l'augmentation de la cytotoxite dépendant des Ig G 2a, l'expression des récepteurs pour les Ig G et la dégranulation de ces cellules.

Cet effet stimulant est retrouvé dans les produits de sécrétion du parasite à tous les stades d'évolution chez l'hôte Vertébré, y compris les adultes des 2 sexes.

L'effet stimulant du facteur actif (thermosensible) est dû à des protéases neutres notamment de spécificité collagénase (possiblité d'utiliser un substrat tel que l'azocoll). Deux activités principales, de poids moléculaire différent ont pû être identifiées.

Le dernier point du travail porte sur l'activité des protéases neutres aux différents stades du développement de *Schistosoma mansoni*.

Ont ainsi été testés les schistosomules, les jeunes schistosomes de 20 jours et les vers adultes. En outre ont également été testés des homogénats totaux ou traité par la saponine-calcium. Des substrats permettant de détecter les endoprotéases, les carboxypeptidases et les amino-peptidases ont été utilisés.

L'utilisation d'inhibiteurs spécifiques a permis de mettre en évidence que l'activité endoprotéase majeure des schistosomules est une sérine protéase tandis que les stades ultérieurs se caractérisent par des activités thiol-protéases. Par contre les aminopeptidases (métallo-peptidase) présentent les mêmes caractéristiques quels que soient les stades envisagés.

Un chapitre de synthèse "Discussion et Conclusion" commente les résultats présentés.

Dans la partie consacrée au clivage des Ig G et à ses incidences sur les macrophages, l'auteur est amené notamment à évoquer le raisonnement et le plan de recherche qui l'ont conduit à parvenir à l'isolement du tripeptide responsable de l'inhibition de l'activité effectrice des macrophages dépendants des Ig E.

Un des points particulièrement intéressants de la deuxième partie de la discussion porte sur la finalité de l'activation des éosinophiles par les facteurs thermosensibles élaborés par les schistosomes. Il montre comment les produits sécrétés par les vers adultes peuvent réguler les mécanismes effecteurs

dirigés contre les larves du parasite et éviter ainsi un processus de réinfection.

Un schéma, très dédactique résume les résultats obtenus.

Le travail de Monsieur Claude AURIAULT présente un intérêt considérable à plusieurs niveaux :

- Dans le domaine de la Biologie Cellulaire, il fournit un modèle intéressant de régulation d'activité cellulaire.

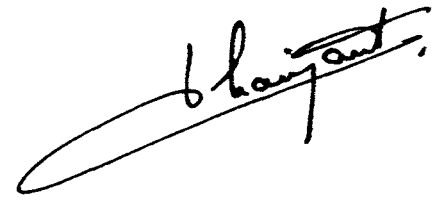
- Sous l'angle de la lutte contre le schistosome, la découverte du tripeptide inhibiteur des activités macrophagiques permet d'envisager des applications pharmacologiques.

- Enfin sous l'angle des mécanismes d'adaptations parasitaires, le travail fournit des hypothèses séduisantes pour expliquer les modalités de survie des parasites et la régulation de leur population.

En conclusion, le rapporteur donne un avis extrèmement favorable à la soutenance de la thèse en vue de l'obtention du titre du Docteur ès-Sciences

A Villeneuve d'Ascq, le 8 septembre 1983

Professeur A. DHAINAUT



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Pierrette CHATEAUREYNAUD

RAPPORT SUR LE MEMOIRE DE THESE

présenté par M. Claude AURIAULT

intitulé :

"Régulation de la réponse immunitaire par le parasite trématode *Schistosoma mansoni*"

en vue de l'obtention du grade de Docteur ès-Sciences Naturelles

Les travaux effectués par M. Cl. AURIAULT, très bien résumés dans son mémoire de thèse, s'inscrivent dans l'ensemble du travail du laboratoire dirigé par Monsieur le Professeur A. Capron.

Depuis plus de 10 ans, les chercheurs de cette équipe ont amené tant dans le domaine de l'immunologie parasitaire des helminthiases que dans celui de l'Immunologie Générale une somme très importante de résultats qui leur ont permis une nouvelle compréhension de certains mécanismes fondamentaux de défense de l'organisme et de leur modulation (nouvelle compréhension du rôle de certains anticorps anaphylactiques, IgE par exemple).

Le travail personnel de M. AURIAULT dans ce groupe a pour objet les relations hôte-parasite dans la Schistosomiase.

Schistosoma mansoni est un ver trématode parasite de l'Homme. On peut évaluer à 300.000.000 le nombre d'individus infestés dans le monde.

Les mécanismes d'infestation d'une part et les relations hôte-parasite d'autre part ont été étudiés sur des rongeurs de laboratoire : souris et rats.

La souris, hôte du schistosome, se montre très sensible à l'infection au point que le ver peut effectuer son complet développement à l'intérieur du Mammifère .

Le rat, par contre, apparaît relativement résistant dans la mesure où, bien que les cercaires soient infestantes et peuvent migrer dans le foie, les parasites sont ensuite éliminés rapidement.

L'ensemble du travail de M. AURIAULT a été fondé sur les réactions du rat infesté par le schistosome.

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La grande originalité de la thèse de M. AURIAULT est de considérer les relations Mammifère-Ver, hôte-parasite dans l'optique de la réponse du parasite à l'hôte.

Le parasite, adapté à son hôte (nous verrons que les protéases secrétées par le parasite ont à la fois une activité spécifique et limitée dans le temps) va tenter de moduler la réponse immunitaire du Mammifère infesté de telle sorte que hôte et parasites puissent continuer à vivre.

Claude AURIAULT a en effet montré que des protéases parasitaires, qu'il a identifiées, caractéristiques de la schistosomule et non de la cercaire, sont capables de cliver les Immunoglobulines G que l'hôte a élaborées contre le parasite et qui se trouvent fixées à sa membrane.

Les IgG sont coupées de telle sorte que la partie variable est libérée sous forme de peptides et que la fraction constante reste fixée à la membrane du parasite.

Les peptides provenant de la partie variable de l'Immunoglobuline G de rat libérés ont différentes propriétés :

A/Ils sont inhibiteurs des fonctions macrophagiques. Cette conclusion de l'auteur est fondée sur l'observation de 3 critères de l'activité macrophagique :

- libération de β glucuronidase
- incorporation de glucosamine
- phagocytose de particules de latex

B/Les peptides clivés par les protéases de Schistosomule ont également la propriété de stimuler les fonctions effectrices des éosinophiles. Cette stimulation est également observée avec des produits de sécrétion de larves plus âgées. Ainsi on met en évidence ici un effet contraire au premier : une cytotoxicité accrue anti-schistosomule, élaborée par l'hôte et IgG dépendante.

C/Le fragment Fc reste fixé sur la membrane du parasite et Cl. AURIAULT précise expérimentalement où se situe la zone de clivage entre Fab et Fc.

Ce chercheur montre que le fragment Fc fixe le C1q, premier élément de la voie classique du complément, permettant la cascade enzymatique de son activation et par suite devant donner sa cytotoxicité à un anticorps.

Dans ce cas précis, il apparaît que le phénomène aboutisse (en l'absence du fragment Fab) à une consommation locale du complément qui ne peut activer un système antigène-anticorps incomplet.

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Ainsi dans les 3 mécanismes découverts par M. AURIAULT et permettant la survie du parasite par modulation de la réponse immunitaire de l'hôte, le parasite clive l'anticorps spécifique de l'hôte, libère des peptides qui inhibent les macrophages, stimulent les éosinophiles. La partie de l'anticorps de l'hôte, restée fixée à la membrane, est un "piège" à complément empêchant ce dernier d'avoir une activité cytotoxique à l'égard des vers et peut-être de produire localement un certain nombre de troubles préjudiciables à l'hôte.

On voit la nouvelle optique amenée par Cl. AURIAULT dans les relations hôte-parasite.

Il est important que ces travaux soient poursuivis-d'une part, comme le souligne l'auteur, dans le domaine des applications des peptides particuliers élaborés par les enzymes parasitaires et modulant la réponse immunitaire des Mammifères.

- d'autre part, en considérant que les vers libres sont capables de sécréter certains chaînons du complément, sont capables de potentialiser les anticorps de vertébrés supérieurs (IgG), ce qui élargit peut-être encore le champ des investigations dans le domaine des relations hôte-parasite.

Le Mémoire de M. AURIAULT est très agréablement présenté, une excellente revue bibliographique où il situe tout l'apport du laboratoire de Monsieur le Professeur Capron auquel il appartient, puis la description de son propre travail.

Il est très appréciable qu'il ait fondé son mémoire sur d'excellentes publications qui lui dont dues, mais surtout qu'il ait résumé brièvement le but, les résultats, les conclusions des publications qu'ils nous donnent, dans une courte page introductive.

On doit également beaucoup apprécier la discussion des résultats, surtout le schéma intégralement original, du aux travaux de Cl. AURIAULT, qui donne une toute nouvelle approche des relations hôte-parasite.

On peut regretter dans un travail aussi remarquable et aussi dense un manque d'effort de simplicité explicative dans la conclusion générale, excellente, mais peut-être un peu trop éloignée du non spécialiste.

En résumé, l'excellent et novateur travail de M. AURIAULT, très bien résumé dans son mémoire, est extrêmement digne d'être soutenu en vue du grade de Docteur ès-Sciences.

Ce travail, tant sur le plan appliqué à l'Immunologie Parasitaire que sur le plan de l'Immunologie fondamentale, amène de nouvelles et remarquables conceptions.

2 Septembre 1983

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THÈSE

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DE LILLE

pour obtenir le grade de

Docteur es-sciences naturelles

par

Claude AURIAULT

Chargé de Recherche au CNRS

RÉGULATION DE LA RÉPONSE IMMUNITAIRE PAR LE PARASITE TRÉMATODE SCHISTOSOMA MANSONI

Présentée le 22 SEPTEMBRE 1983 devant la commission d'examen

Membres du jury : Président : M. M. DURCHON

Rapporteurs : M. A. CAPRON
Mme P. CHATEAURAYNAUD
M. A. DHAINAUT

Examinateurs : M. J. LOUIS
M. G. TORPIER

Cette thèse est dédiée à

Martine

David

Marie

Mes parents

Mes beau-parents

Toute ma famille

Le parlage des gens fleure bon le terroir
En ces lieux où naguère on pouvait percevoir
le bruissement discret d'une humble kichenotte
Lithamisant un frais visage aux yeux de jais
Je ne sais quel miracle introduit une note
De luminosité dans l'azur saintongeais.

Guy Palissière

(*extrait de "Chateauneu"*
revue de la Société d'Etude Folklorique
du Centre Ouest - Tome XIII -
Mai - Juin 1979).

Ce travail a été réalisé dans le laboratoire et sous la direction du Professeur André CAPRON

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Monsieur le Professeur CAPRON

J'éprouve une grande fierté et je suis très honoré de faire partie de votre équipe. Je tiens à vous exprimer ici toute ma gratitude et mon amitié.

J'exprime mes sentiments de gratitude et mon profond respect à Monsieur le Professeur DURCHON et aux membres du jury.

Pour tous mes amis du laboratoire qui de près ou de loin m'ont aidé de leurs mains ou de leur savoir : MERCI.

Tout chercheur, je crois, se souvient comme de son premier amour, de son premier contact avec une vérité neuve si humble qu'ait pu être celle-ci.

Jean Rostand.

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INTRODUCTION

Comment expliquer la survie des parasites et plus particulièrement des helminthes pendant des mois voire même des années chez des hôtes immunologiquement compétents ?

Cette question est sûrement la plus fondamentale que se posent les immunologistes travaillant sur des modèles parasitaires depuis de nombreuses années. Les progrès remarquables réalisés récemment ont mis en évidence les mécanismes de destruction succeptibles d'intervenir dans la réponse humorale ou cellulaire acquises au cours de l'infection. Toutefois malgré l'apparente efficacité et la diversité des mécanismes mis en oeuvre, le parasite peut se maintenir dans l'organisme même si dans certaines situations, il est partiellement rejeté. La réciproque évidente est que des individus parasités par des helminthes peuvent survivre de nombreuses années comme si le parasite ménageait, voire peut-être protégeait, le mammifère l'hébergeant. C'est dans cette vaste perspective de l'étude des relations hôte-parasite dans le mode d'infection par Schistosoma mansoni que nous avons entrepris une série de travaux dont une partie est décrite dans cette thèse.

CARACTERISTIQUES GENERALES DE L'INFECTION PAR SCHISTOSOME

La schistosomiase est une infection qui affecte 300 millions de personnes dans le monde. Des modèles expérimentaux sur diverses espèces animales ont permis la réalisation de nombreuses recherches

concernant l'immunorégulation au cours des infections parasitaires. L'infection par Schistosoma mansoni est caractérisée par la présence de vers adultes dans les veines portes et mésentériques à la suite de mécanismes complexes de migration déclenchés par la pénétration transcutanée des cercaires qui sont les larves infestantes. Ces cercaires provenant de l'eau douce se transforment en schistosomules après pénétration chez l'hôte. L'utilisation des modèles d'infection expérimentale du rongeur tels que la souris et le rat ainsi que le maintien en culture des schistosomules a permis l'analyse plus précise des mécanismes immunologiques au cours de cette infection.

a) Spécificités d'espèce et intraspécifique

Selon les différentes espèces animales étudiées, le degré de résistance à l'infection par S. mansoni est variable allant d'une résistance totale jusqu'à la mort des hôtes avec tous les intermédiaires possibles. Il semble en effet exister trois catégories d'hôtes suivant la succeptibilité à l'infection par schistosomes. Ceux qui sont des hôtes totalement résistants, ceux qui peuvent être infectés mais qui sont de mauvais hôtes et ceux qui sont succeptibles (Kagan, 1958). Le hamster et la souris par exemple sont considérés comme les hôtes les plus sensibles. Ils permettent au parasite de se développer jusqu'au stade adulte de s'accoupler et de pondre des oeufs. Le rat par contre est un hôte dit semi-permissif dans la mesure où les cercaires peuvent l'infester et même accomplir le stade de migration hépatique.

Mais à partir de la 3ème à la 4ème semaine de post-infection commence la phase de rejet des parasites aboutissant à une diminution et même une disparition de la masse parasitaire (Smithers et Terry, 1965 ; Maddison et Coll., 1970). Cette résistance à l'infection est suivie par une immunité à la réinfection importante et prolongée. L'homme se comporte comme un hôte susceptible. On peut observer des infestations de très longues durées restant d'ailleurs souvent stables dans les populations d'âge adulte.

Il semble qu'en plus des spécificités d'espèce, il y ait des différences de susceptibilité à l'intérieur d'une espèce que l'on appelle spécificité intraspécifique observée chez l'homme (Cardoso, 1953) ou dans les modèles expérimentaux (Kagan, 1958).

b) L'immunité au cours de la schistosomiase expérimentale (modèle expérimental : Rat)

1) La réponse humorale

Au cours de l'infection,des anticorps apparaissent en assez grande quantité dans le sérum des animaux parasités. Les anticorps apparaissent assez tardivement ou plus exactement le maximum du taux d'anticorps chez le rat est obtenu aux alentours de la 12ème semaine alors que le rat est immun dès la 4ème semaine (Maddison et coll., 1970). Tous les résultats expérimentaux obtenus soit par transfert passif de sérum d'animal infesté (Rousseaux et Coll., 1980) ou chez des animaux traités par des anti-μ montrent que la réponse anticorps est un facteur essentiel de l'immunité anti-schistosome (Bazin et coll., 1980).

Toutefois, les anticorps seuls sont insuffisants semble-t-il pour assurer une protection significative. Leur rôle sera décrit dans les chapitres consacrés à la description des mécanismes effecteurs.

Bien que tous les isotypes soient représentés au cours de l'infection par S. mansoni, la réponse IgE mérite une attention particulière. En effet dans la schistosomiase humaine ou expérimentale, les taux d'IgE sériques sont considérablement augmentés et associés à une production importante d'anticorps IgE-spécifique du parasite (Dessaint et coll., 1975 ; Rousseaux-Prévost et coll., 1978). Le fait important est que chez le rat, une corrélation nette est observée entre le développement de l'immunité à la réinfection et la réponse IgE spécifique du parasite (Rousseaux-Prevost et coll., 1978). De plus, chez le rat comme chez la souris, on observe une production importante d'anticorps anaphylactiques non réaginiques de sous classe respectivement IgG2a ou IgG1 (Rousseaux et coll., 1980 ; Santoro et coll., 1978). Contrairement à ce que l'on pourrait croire, cette importante production d'anticorps anaphylactiques n'est pas associée à des manifestations allergiques. Le contraire même a été reporté d'une association négative entre l'allergie et le parasitisme dans les zones d'endémie (Anderson, 1974).

2) La réponse cellulaire et l'hypersensibilité retardée

Au cours de la schistosomiase on peut observer une réponse cellulaire dont les manifestations pourraient être soit favorables soit néfastes pour l'hôte. Les réactions que nous décrivons dans ce chapitre seront les réactions d'hypersensibilité retardées. Celles-ci peuvent être décrites comme des réactions cellulaires d'une sensibilité accrue à des antigènes spécifiques, en l'occurence les antigènes des schistosomes, dont les manifestations ne se produisent qu'après un certain temps de contact avec ces antigènes. Des degrés variables de ces réactions cellulaires dirigées contre les schistosomules peuvent être observées au niveau de la peau, où on peut assister à une infiltration inflammatoire consécutive à un afflux de leucocytes polymorphonucléaires ainsi qu'au niveau des poumons et des ganglions lymphatiques. La réaction cellulaire la mieux étudiée a été la réaction granulomateuse autour de l'oeuf de schistosome. Sa formation est consécutive à la libération d'antigènes par les oeufs. Il est admis que les réactions pathologiques observées au cours de la schistosomiase sont en grande partie dues à la formation de ce granulome inflammatoire qui est une des réactions cellulaires les plus typiques de la bilharziose (Domingo et Warren, 1967, 1968). L'hypersensibilité retardée de type granulome peut être induite par l'injection d'oeufs de schistosomes et peut être transférée passivement d'un animal infecté par S. mansoni à un animal non infecté

par des cellules ganglionnaires ou spléniques mais non par le sérum (Von-Lichtenberg et coll., 1962, 1963). Boros et Warren (1970) ont montré que le matériel antigénique soluble de l'oeuf peut en petite quantité induire une réaction d'hypersensibilité retardée granulomateuse sans induire de formation d'anticorps détectables par la technique d'hémagglutination. La technique de formation de granulomes in vitro a pu mettre en évidence le recrutement de macrophages, lymphocytes, éosinophiles et fibroblastes autour de l'oeuf et a clairement montré la spécificité immunologique de la réaction (Doughty et Phillips, 1981).

3) L'immunité concomitante

Smithers et Terry (1969) ont été les premiers à émettre l'hypothèse que l'immunité acquise peut avoir un rôle important dans la schistosomiase. Le terme de "concomitant" a été adopté par analogie avec l'immunité anti-tumorale ou la greffe d'une seconde tumeur sur des animaux n'est pas acceptée si ceux-ci portent une première tumeur. Au cours de l'infection par schistosomes le ver adulte semble être le stimulus majeur de l'immunité, En effet, au cours d'une réinfection, alors que le ver adulte n'est pas affecté par la réponse immune qu'il a lui-même suscitée, les jeunes larves par contre sont détruites. Le mécanisme de l'immunité concomitante tel qu'il a été mis en évidence chez le singe Rhésus permet d'expliquer en partie au moins la longue persistance des infections par schistosome, grâce au maintien d'une charge parasitaire à peu près stable. On peut même assister

à une diminution du nombre d'oeufs avec l'âge de l'infection. Toutefois, d'autres mécanismes peuvent également intervenir au cours de la réponse immune, comme des réactions faisant intervenir notamment des mécanismes non spécifiques.

L'existence d'un état d'immunité nous amène donc à décrire les différents mécanismes effecteurs étudiés ayant comme cible principale le schistosomule.

c) Les mécanismes effecteurs

Dans la plupart des autres maladies parasitaires tout comme dans la schistosomiase, la réponse immunitaire fait intervenir divers composants du système immunitaire comme les anticorps de différents isotypes, des facteurs complémentaires et différentes populations cellulaires pouvant agir ensemble ou indépendamment à des temps donnés de l'infection. Compte-tenu de la complexité de la situation in vivo, l'analyse des mécanismes effecteurs a été effectuée en grande majorité par des études in vitro qui ne peuvent bien sûr pas rendre compte de l'ensemble des mécanismes considérés. Par contre, ces approches ont le grand avantage de pouvoir dissocier les différents composants intervenant dans le mécanisme de cytotoxicité.

1) Cytotoxicité par anticorps léthaux

Il a été mis en évidence que des sérums immuns de Singe Rhésus ou humains en présence de complément frais de cobayes peuvent être léthaux pour les schistosomules (Clegg et Smithers, 1972 ; Capron et coll., 1974, 1977). Les anticorps impliqués ont été identifiés comme étant des IgG chez ces deux espèces

mais également chez d'autres espèces étudiées comme le lapin, le rat et la souris, comme nous l'avons déjà mentionné. La pertinence biologique de ces anticorps cytotoxiques reste toutefois à préciser. En effet, la nécessité d'une source de complément hétérologue alors que plusieurs exemples montrent l'inefficacité du mécanisme en présence d'un sérum homologue est une des principales objections apportée à ces résultats. Par ailleurs, le maintien d'un taux élevé de ces anticorps dans les sérums à des moments où l'immunité décline, montre qu'en tout cas les anticorps léthaux ne représentent pas les seuls mécanismes immunitaires développés par l'hôte pour se protéger des schistosomes. En effet, des essais de protection de souriceaux par infection expérimentale de mères n'ont pas abouti ce qui a suggéré aux auteurs (Weinman, 1960 ; Taylor et coll., 1971) le peu de rôle joué par les anticorps seuls, dans la protection.

2) Cytotoxicité cellulaire

Avant d'aborder plus en détail les mécanismes effecteurs à médiation cellulaire principalement étudiés, impliquant des cellules phagocytaires comme les macrophages et les éosinophiles, il est nécessaire de mentionner le cas particulier des lymphocytes. En effet quoique de nombreux arguments expérimentaux montrent clairement la thymo-dépendance de la réponse anti-schistosome, il a été démontré que des cellules T cytotoxiques (CTL) ne participent pas aux mécanismes d'élimination des parasites (Butterworth et coll., 1979). Récemment, une équipe a décrit un mécanisme de cytotoxicité in vitro impliquant la participation de T lymphocytes et particulièrement de CTL (ELLNER et coll., 1982) mais l'impossibilité de reproduire ces résultats dans plusieurs laboratoires dont le nôtre, leur retire une partie de leur crédibilité (Communications personnelles).

Pourtant, l'hypothèse d'une cytotoxicité par CTL apparaissait d'autant plus évidente qu'il a été montré que les schistosomules peuvent acquérir à leur surface des produits K/D ou I du complexe majeur d'histocompatibilité de la souris (Sher et coll., 1978) ce qui est en principe une condition nécessairement requise pour qu'une activité CTL soit possible. L'adhérence de lymphocytes alloréactifs de phénotype Lyt 1^- 2 3^+ sur des schistosomules n'est pas suffisante pour tuer la larve puisque les cellules se détachent après quelques heures de contact (Butterworth et coll., 1979). Différentes explications peuvent être apportées pour expliquer cette inefficacité des CTL à lyser les larves et seront discutées dans un chapitre ultérieur.

- Cytotoxicité des neutrophiles

Dean et coll. (1975) ont mis en évidence que des neutrophiles de cobaye mis en présence de sérums homologues sont capables de tuer des schistosomules in vitro. Dans le cas de ces expériences, le fait de rajouter des macrophages ou des éosinophiles n'augmente pas le taux de cytotoxicité et les auteurs suggèrent que les neutrophiles représentent une des principales cellules impliquées dans l'immunité anti-schistosome en coopération avec des IgG2 spécifiques. Ces travaux ont été largement critiqués car les sérums utilisés sont des sérums déjà cytotoxiques en eux-mêmes. Par ailleurs, la classe d'anticorps en cause fixe le complément et la participation des neutrophiles dans ce cas n'est plus nécessaire pour assurer une cytotoxicité. Des études plus récentes contestent ces résultats et montrent que les neutrophiles, bien que capables d'adhérer sur les schistosomules ne dégranulent pas sur la cible et ne lui causent aucun dommage (David et coll., 1980). Le débat reste ouvert,toutefois les travaux effectués dans notre laboratoire montrent plutôt une

certaine inefficacité des neutrophiles pour tuer les schistosomules et quelques arguments allant dans ce sens sont développés dans le chapitre des résultats.

- Cytotoxicité des macrophages IgE-dépendante

Des macrophages normaux, incubés avec du sérum de rat immun vis-à-vis des schistosomes, adhèrent fortement et tuent in vitro les schistosomules de S. mansoni. La mise en évidence de la thermolabilité ainsi que l'indépendance vis-à-vis du complément de ce facteur sérique ont conduit à démontrer que des anticorps de nature IgE sont impliqués dans la réaction de cytotoxicité. Des techniques biochimiques d'immuno-adsorption avec des anti-immunoglobulines de rats ont confirmé ces premiers résultats (Capron et coll., 1975 ; Capron et coll., 1977 ; Joseph et coll., 1977). Différentes expériences d'inhibition ont également permis d'écarter l'hypothèse de la participation d'autres isotypes comme les IgG ou les IgM. Le mécanisme fait donc intervenir la fixation cytophile préalable de l'IgE immune sur un récepteur spécifique pour cette immunoglobuline sur la membrane des macrophages (Dessaint et coll., 1979). Une étude plus précise, utilisant l'IgE purifiée aggrégée par des procédés chimiques a mis en évidence que les dimères d'IgE représentent la forme minimale d'aggrégation qui déclenchent l'activation des macrophages induisant ainsi leur effet toxique (Capron et coll., 1977). Des expériences similaires ont été effectuées utilisant des sérums d'infections par S. mansoni et des monocytes humains ainsi que des macrophages péritonéaux de babouins (Joseph et coll., 1978). Dans ces systèmes également, les IgE sous forme complexée stimulent le mécanisme cytotoxique des phagocytes vis-à-vis des schistosomules.

La spécificité anti-parasitaire des IgE dans la réaction de cytotoxicité a été démontrée par absorption d'un sérum de rat immun par des antigènes de S. mansoni. Celle-ci correspond à une décroissance de la cytotoxicité des macrophages IgE-dépendante d'une manière dose-dépendante avec la concentration d'antigène utilisée. L'utilisation d'allergènes non parasitaires ou d'antigènes d'autres espèces de schistosomes tels que S. bovis en aucun cas ne diminuait le taux de mortalité des larves (Capron et coll., 1976).

La cytotoxicité IgE dépendante des macrophages peut être décrite en deux étapes successives ; la première impliquant la liaison sur son récepteur cellulaire de l'IgE sous forme aggrégée, au moins sous forme de dimères. Cette étape conduit à une activation de la cellule, indépendamment de la spécificité des IgE. La seconde se caractérise par une adsorption spécifique des macrophages sur leurs cibles. Cette étape conduit à la mort des larves en 6 à 18 heures et nécessite la présence sur le macrophage d'IgE spécifiques d'antigènes exprimés à la surface des schistosomules. Dans le chapitre consacré aux mécanismes effecteurs non spécifiques nous décrivons d'autres mécanismes de cytotoxicité in vitro impliquant les macrophages sans la présence d'anticorps. Ceci pour souligner la part importante que semble occuper le macrophage dans l'immunité anti-schistosome.

- Cytotoxicité des éosinophiles anticorps-dépendante

L'observation initiale de Butterworth et coll. (1975) a mis en évidence le rôle des éosinophiles comme cellules effectrices vis-à-vis des schistosomules en présence d'anticorps spécifiques. Ces auteurs ont en effet montré que des éosinophiles humains normaux peuvent tuer in vitro des schistosomules en

présence d'IgG de patients infestés par S. mansoni et ceci en l'absence d'une source de complément. Ces études corroboraient une étude pathologique préalable qui mettait en évidence l'accumulation d'éosinophiles autour des larves consécutivement à la pénétration transcutanée (Hsü et coll., 1971 ; Von Lichtenberg, 1976). De plus, Mahmoud et ses collègues (1975) rapportaient qu'un sérum anti-éosinophile injecté à des souris déprimait l'immunité à la réinfection ce qui a motivé un certain nombre d'études des fonctions effectrices de cette population cellulaire au cours des parasitoses à helminthes notamment celles que Monique Capron a effectuées dans notre laboratoire.

Chez le rat, M. Capron a notemment mis en évidence le mécanisme de cytotoxicité par éosinophiles en identifiant les anticorps responsables comme étant des IgG2a, qui est la sous-classe majeure des IgG dans cette espèce, dotée d'activité anaphylactique non réaginique, mais également plus récemment des IgE (Capron et coll., 1978 ; Capron et coll., 1981). On peut observer dans ce modèle l'association des éosinophiles avec des mastocytes en étroit contact avec la cible bien que les mastocytes en eux-mêmes soient incapables de tuer les larves. Ces mastocytes jouent le rôle de cellules accessoires dans la réaction de cytotoxicité puisque des populations purifiées d'éosinophiles dépourvues de mastocytes ont très peu d'effet cytotoxique en présence de sérum de rats immuns (Capron et coll., 1978). La cytotoxicité des éosinophiles peut être restaurée par l'addition des produits de dégranulation des mastocytes. Ceci suggère une dégranulation mastocytaire à l'aide des IgG2a et des IgE spécifiques au contact de la larve et une diffusion de ces produits vers les éosinophiles comme facteurs coopératifs. Parmi ces facteurs mastocytaires, les

tétrapeptides synthétiques à activité chimiotactique pour l'éosinophile (ECF-A) jouent un rôle prédominant. Une corrélation a été montrée entre l'ECF-A (Ala-Gly-Ser-Glu) et ses analogues (Val-Gly-Ser-Glu, Val-Pro-Ser-Glu) et l'expression des récepteurs Fc des éosinophiles (Capron et coll., 1981). Ces observations ainsi que l'augmentation de l'expression des récepteurs C3b par les ECF-A tétrapeptides (Anwar et Kay, 1977) mettent en évidence que les facteurs chimiotactiques outre leur activité sur la migration cellulaire peuvent également intervenir au niveau de l'expression de récepteurs membranaires. Dans ce modèle, l'activité cytotoxique des éosinophiles semble déclenchée par deux signaux: L'un résulte de la fixation des anticorps spécifiques(préfixés sur le schistosomule) sur le récepteur spécifique des classes IgG2a ou IgE. Le second par des produits d'activation provenant de la dégranulation des mastocytes, celle-ci résultant de l'interaction de la cellule avec les mêmes anticorps anaphylactiques.

Ainsi, deux mécanismes distincts de la cytotoxicité par éosinophiles semblent opérationnelles a des périodes différentes au cours d'infections expérimentales chez le rat. L'une avant six semaines d'infection dont le rôle peut être attribué aux anticorps de type IgG2a, l'autre après 8 semaines d'infection attribuable essentiellement aux anticorps de type IgE. Ceci pose le problème particulier de l'implication des anticorps de type anaphylactiques dans la protection anti-schistosome et peut-être plus généralement anti-helminthes.

3) Les anticorps anaphylactiques et les mécanismes effecteurs

Dans ces deux mécanismes de cytotoxicité cellulaire anticorps dépendants vis-à-vis des schistosomules de S. mansoni, la participation essentielle d'anticorps anaphylactiques est donc mise en évidence. Des résultats expérimentaux dans d'autres modèles parasitaires comme les filarioses ou les infections à Trichinella chez les rongeurs ont également montré l'implication d'anticorps anaphylactiques dans les mécanismes de cytotoxicité par des cellules phagocytaires (Haque et coll., 1981). Outre les données expérimentales obtenues chez le rat, des travaux réalisés chez la souris ont également montré que les IgG1 qui constituent la sous classe anaphylactique non réaginique de cette espèce sont également impliquées dans un processus de cytotoxicité des éosinophiles vis-à-vis des schistosomules. Ceci suggère donc que outre leur participation au processus classique d'hypersensibilité immédiate, les anticorps anaphylactiques peuvent également exercer une autre fonction comme inducteur de l'activation phagocytaire ce qui aura pour conséquence la libération d'enzymes et autres molécules lysosomiales responsables de l'effet toxique sur leurs cibles.

Une preuve indirecte du rôle protecteur des anticorps anaphylactiques in vivo a été apportée par des transferts passifs de sérums dont la déplétion en anticorps anaphylactiques a été effectuée par immunoadsorption. Dans ce cas, l'immunité transférrée diminue significativement quand les sérums sont diminués en anticorps de type IgG2a et IgE anti-schistosomes aux périodes où ils sont impliqués dans les mécanismes de cytotoxicité in vitro (Capron et coll., 1980).

Ainsi les modèles parasitaires nous aident à mieux comprendre le rôle biologique des anticorps anaphylactiques et leur participation dans l'immunité protectrice (Capron et Dessaint, 1977).

4) Les mécanismes effecteurs non spécifiques

A côté des mécanismes de cytotoxicité dont la spécificité sera en liaison avec les anticorps dont ils dépendent, il existe différents mécanismes qualifiés de non spécifiques. Bout et Coll. (1981) ont notamment mis en évidence qu'après l'activation par une lymphokine telle que le MIF (Macrophage inhibitory factor), les macrophages normaux de rat deviennent cytotoxiques pour les schistosomules. Nous avons pu montrer d'autre part que ces mêmes macrophages pré-activés par des IgG non spécifiques aggrégées peuvent tuer les schistosomules. Mais dans ce cas la présence d'une source de complément est nécessaire. Ces observations indiquent donc que différents mécanismes d'activation des macrophages sont susceptibles d'intervenir dans l'expression de la cytotoxicité de ces cellules parallèlement ou à des moments différents du mécanisme de cytotoxicité IgE dépendant décrit précédemment, au cours des infections par schistosomes.

Les éosinophiles et peut être les neutrophiles pourraient être également impliqués dans la réaction de cytotoxicité non spécifique en présence d'une source de complément (Incani et Mc Laren, 1981). Par contre, aucune démonstration d'activité des cellules NK dans l'immunité anti-parasite n'a été apportée et le taux de cytotoxicité par des cellules normales reste toujours extrêmement bas (ELLNER et MAHMOUD, 1979).

Il a été montré dans notre laboratoire que l'activation du complément par la voie alterne peut endommager irréversiblement la membrane des larves de schistosomes (Santoro et coll., 1979). Cet effet cytocide du complément peut être amplifié lorsque des cellules possédant un récepteur C3b, comme les éosinophiles, sont activées en présence de complément (Ottesen et coll., 1977 ; Ramalho-Pinto et coll., 1978).

La plupart des mécanismes ainsi décrit mettent en évidence la potentialité cytotoxique de différents mécanismes dépendants du complément, de cellules immunocompétentes ou des deux. Mais ces travaux restent souvent très descriptifs. Toutes ces approches de l'immunité non spécifique anti-parasite mériteraient de faire l'objet d'études plus approfondies afin de déterminer plus précisément les composants intervenant, à des niveaux et de manières différentes.

d) Les mécanismes d'évasions et d'immunorégulations développés par les parasites

Quelle que soit l'efficacité des mécanismes effecteurs décrits dans les chapitres précédents, il faut bien admettre que dans les conditions naturelles, ces mécanismes effecteurs ne peuvent s'exprimer pleinement et que les parasites doivent donc détourner la réponse immune qu'ils ont eux-mêmes déclenchée. Il existe vraisemblablement une grande différence entre la source des connaissances actuelles sur les mécanismes d'évasions et la réalité biologique mais les travaux faisant l'objet de cette thèse s'inscrivent dans ce grand chapitre et nous ne citerons que les mieux étudiés avec un regard plus particulier sur les mécanismes

d'immunorégulation au cours de la schistosomiase.

Il est donc nécessaire de distinguer les mécanismes d'évasions opérant au niveau des schistosomules, qui donc permettent la survie des parasites après leur pénétration chez l'hôte, des mécanismes de régulation en relation avec les vers adultes qui interviendront sur la réponse immune de l'hôte.

Les questions qui se posent sont donc :

1) Comment le ver adulte se protège et régule la réponse immunitaire qu'il suscite ?
2) Comment s'exerce cette réponse sur le schistosomule cible ?
3) Quelles sont les possibilités des schistosomules de se protéger et de réguler la réponse immunitaire ?

1) Modulation membranaire

Torpier et Coll. (1979) ont mis en évidence que des ligands, incluant les anticorps, peuvent induire une réaggrégation des protéines intégrales de la membrane du schistosome. Bien qu'en présence du complément, et en présence de certains anticorps, ce mécanisme de "copatching" peut conduire à la destruction du parasite, il peut être suivi en l'absence de complément par un "cocapping" et une élimination des aggrégats au niveau des épines tégumentaires. Ceci met en évidence que le parasite, après la liaison de ligands immunologiques peut restituer l'intégrité de sa membrane (Torpier et coll., 1980) au moins par un turnover membranaire rapide.

2) Acquisition d'antigènes d'hôtes

Damian (1964, 1967) et Capron et coll. (1965, 1968) ont été les premiers à démontrer l'existence chez le schistosome de déterminants antigéniques identiques à ceux de l'hôte vertébré. A partir de ces observations initiales, l'existence de ces antigènes d'hôte a été démontré chez d'autres trématodes ainsi que chez des cestodes et des nématodes (Capron et Coll., 1968). Ceci suggèrait que ces antigènes pourraient jouer un rôle important dans l'adaptation des parasites à leurs hôtes. Clegg et coll. (1971) démontrent que les antigènes d'hôte sont acquis par les schistosomules durant les 15 premiers jours qui suivent la pénétration chez l'hôte et que certains d'entre eux au moins sont associés aux membranes érythrocytaires. Par des réactions d'agglutination mixte , Damian (1974) met en évidence la présence de déterminants identiques à l'α 2 macroglobuline de souris à la surface des schistosomes. Plus récemment, des immunoglobulines ont été mises en évidence à la surface des vers adultes (Kemp et coll., 1977) ainsi que des déterminants antigéniques controlés par les régions K et I du système majeur d'histocompatibilité de la souris (Sher et Coll., 1978).

D'autres observations identiques ont été faites par de nombreux auteurs concernant d'autres antigènes d'hôtes et il semble donc que les hypothèses concernant l'immunoadaptation, développées par Capron et coll. en 1968 et n'excluant pas l'intervention d'autres mécanismes, soient confirmées. D'un point de vue immunologique il est concevable que l'existence de tels antigènes à la surface des schistosomes puissent au moins partiellement protéger le parasite contre la réponse immune de l'hôte. Toutefois, la pertinence fonctionnelle de ces antigènes d'hôte reste à démontrer bien qu'il semble

que l'acquisition des antigènes d'hôtes par les schistosomes est en corrélation avec l'acquisition de leur résistance aux mécanismes effecteurs (Clegg et Smithers, 1972).

3) Accepteurs de surface des schistosomules

Par des techniques de formation de rosette, il a été mis en évidence des accepteurs à la surface des schistosomules pour des immunoglobulines et des composants du complément. En effet, la présence d'accepteurs pour le C1q (Santoro et coll., 1980) et C3b (Ouaissi, et coll., 1980) disparaissant avec l'évolution du parasite a été notamment décrite. La fonction exacte de ces récepteurs n'est pas encore clairement établie mais ces observations sont à corréler avec le fait que des schistosomules peuvent activer le complément par les voies classiques et alternes bien que les récepteurs en eux-mêmes ne semblent pas intervenir dans l'activation. Par contre, les travaux récents de Fearon (1980) suggèrent au contraire que ces structures pourraient intervenir comme inhibiteurs de la cascade de l'activation du complément ce qui peut représenter un système d'évasion aux mécanismes effecteurs dépendant du complément.

Nous apporterons une mention toute particulière à l'exisrence d'un accepteur pour les immunoglobulines de type G et la β 2-microglobuline compte-tenu des conséquences de l'observation initiale de TORPIER et coll. (1979) sur les travaux faisant l'objet de cette thèse. Lorsque des schistosomules sont incubés en présence d'IgG de différentes espèces animales et humaines, ou simplement leur fragment Fc, une totale inhibition de la formation de rosettes EA est observée. Par contre, l'incubation avec des fragments F (ab)'2 ne modifie pas la fixation des hématies. Cet accepteur est

caractéristique du stade "jeunes schistosomules" puisque ni des cercaires ni des schistosomules de 4 jours récoltés dans les poumons ne fixent d'IgG. Sa sensibilité à la pronase semble indiquer que la structure acceptrice est de nature protéique bien que la trypsine n'exerce aucun effet. Ces observations indiquent donc l'existence d'un accepteur pour les IgG à la surface des schistosomules qui peut être assimilé aux récepteurs Fcγ décrits chez les cellules eucaryotes. La polarité d'apparition de ces accepteurs pour les IgG sur les larves après la rupture de la queue de la cercaire, de la partie antérieure vers la partie postérieure indique bien que leur apparition est un procédé dynamique. Par contre, la β2-microglobuline n'inhibe pas la fixation d'IgG sur les récepteurs des cellules d'eucaryotes alors qu'elle l'inhibe sur la structure acceptrice des schistosomules, ce qui constitue une différence essentielle entre les deux systèmes. Ceci indique donc qu'une même structure membranaire peut fixer aussi bien les IgG que la β2-microglobuline. La fonction biologique de cet accepteur pour les IgG fait l'objet d'une grande partie de mes travaux personnels dont les résultats sont exposés sous le chapitre "Modulation des macrophages par des peptides d'IgG libérés après clivage par des protéases sécrétées par les schistosomules de S. mansoni".

4) Modulation de la réponse immune par les complexes immuns

Dans différentes infections, parasitaires ou non, les complexes immuns jouent un rôle régulateur de la réponse immunitaire. Pour ce qui concerne la schistosomiase, il a été montré que des éosinophiles prélevés à différents temps d'infection sont cytotoxiques in vitro pour les schistosomules sans nécessiter l'addition d'anticorps dans le système. Des études par la technique

de formation de rosette a montré la présence d'anticorps cytophiles à la surface de ces éosinophiles cytotoxiques (Capron et coll., 1979). Or, à certaines périodes après infection, essentiellement après une dizaine de semaines, les éosinophiles de rats immuns sont incapables de tuer des larves même si des anticorps spécifiques leur sont ajoutés. A cette période, les récepteurs Fc sont bloqués tout comme le sont des éosinophiles normaux incubés avec des sérums de cette période particulière (plus particulièrement le culot d'ultracentrifugation de ces sérums) de l'infection par schistosomes. Par contre, à d'autres périodes notamment après 6 semaines d'infection, les sérums n'induisent pas une telle inhibition de la cytotoxicité par éosinophile. Ces expériences indiquent donc que la fonction effectrice des éosinophiles n'est pas constante au cours de l'infection mais peut être modulée à certaines périodes par des complexes immuns (Capron et coll., 1979).

5) Modulation de l'activité cellulaire par des facteurs sécrétés par les schistosomes adultes

L'action de facteurs parasitaires sur la modulation de la réponse immunitaire a été étudiée dans différents modèles. L'existence, notamment de facteurs chimiotactiques pour les éosinophiles d'Anisakis (ECF-Ani) a été décrit par Torisu et coll. (1980). Cette activité est due à des produits d'excrétion-sécrétion du parasite, thermo-sensible, acido-résistant. Au moins deux molécules semblent impliquées, de poids moléculaires 43 000 et 5000 et sont spécifiques des éosinophiles. D'autres études ont montré des activités immunosuppressives dans des sérums de souris infectés par différents parasites comme par exemple par Trypanosoma cruzi (Kuhn et coll., 1980) sans que la participation directe de facteurs parasitaires ait été montrée.

Un produit de sécrétion des schistosomes adultes dénommé SDIF (Schistosome Derived Inhibitory Factor) a été décrit dans notre laboratoire comme ayant des activités inhibitrices de la prolifération lymphocytaire et de la dégranulation mastocytaire (Mazingue et coll., 1980). Il a en effet été montré que la fraction dialysable des produits d'incubation de schistosomes inhibe la prolifération de lymphocytes préalablement stimulés par des mitogènes ou des antigènes de S. mansoni (Camus et coll., 1979). D'autre part, un ou des facteur(s) de la même fraction de poids moléculaire 500 à 1000, thermostable(s), TCA soluble(s) inhibe(nt) fortement la dégranulation in vivo et in vitro de mastocytes stimulés par des composés chimiques ou des réactions anaphylactiques. Les mastocytes comme nous l'avons déjà mentionné sont des cellules accessoires essentielles dans les mécanismes de cytotoxicité par éosinophiles. Quand le SDIF est ajouté dans le système de cytotoxicité impliquant des mastocytes, des éosinophiles et des anticorps, une très importante diminution de la mortalité des larves peut être observée. La purification des facteurs biologiques actifs est actuellement en cours afin de préciser leur mode d'action au niveau cellulaire mais déjà ces études permettent de mettre en évidence l'effet direct des facteurs parasitaires à différents niveaux de la réponse immunitaire. Une partie des résultats qui seront présentés dans les chapitres suivants montreront l'effet régulateur des protéases sécrétés par les schistosomes sur les fonctions cytotoxiques anti-parasite des éosinophiles.

TRAVAUX PERSONNELS

L'enquête scientifique commence toujours par l'invention d'un monde possible ou d'un fragment de monde possible.

P. Medawar

Quelles étaient les questions posées lorsque ce travail fut entrepris ?

Lorsque nous avons commencé cette étude, l'accepteur pour les IgG sur les schistosomules venaient d'être décrit (Torpier et coll., 1979). Ces mêmes auteurs avaient observé que l'aptitude des larves à former des rosettes EA (Erythrocyte anti-erythrocyte) disparaissait après 3 heures de contact entre les parasites et les IgG. Ceci laissait supposer que :

- soit le complexe accepteur-IgG était libéré par les larves ;
- soit seulement les IgG étaient libérées par les larves en maintenant en place le récepteur ;
- soit seulement la portion F(ab')2 de la molécule était clivée de la molécule d'IgG par une hydrolyse enzymatique ce qui laissait supposer que la portion Fc de la molécule restait en place sur les schistosomules.

Quel que soit le cas, la question qui se posait était : "Pourquoi un tel mécanisme ?". L'acquisition d'antigènes d'hôte comme les IgG par les schistosomules peut trouver une explication, mais pourquoi des IgG et non les autres isotypes ? Enfin, compte-tenu des similitudes avec les récepteurs Fc décrits sur les cellules d'eucaryotes et plus particulièrement ceux décrits sur les cellules impliquées dans l'immunité, le rôle fonctionnel éventuel

que peut jouer un accepteur[1] pour des IgG notamment sur la régulation de la réponse immune dirigée contre le parasite devrait être l'orientation essentielle de nos travaux.

(1) Bien que le terme "Récepteur" soit fréquemment employé concernant le site de fixation des IgG à la surface des schistosomes, nous emploieront volontairement dans cette thèse le terme "Accepteur" dans la mesure où sa caractérisation comme "Récepteur" est encore en cours.

ARTICLE n° 1 : CLIVAGE PROTEOLYTIQUE DES IgG FIXEES SUR L'ACCEPTEUR Fc DES SCHISTOSOMULES DE S. MANSONI.

Dans cet article, nous avons abordé les deux questions suivantes :

1) Les IgG après leur fixation sur la membrane du parasite sont-elles éliminées passivement ou clivées par des enzymes protéolytiques parasitaires ?

2) Dans ce second cas, comment s'opère le clivage ?

Pour répondre à ces questions, nous avons dans un premier temps utilisé les mêmes techniques que celles décrites par l'identification de l'accepteur par les IgG, les techniques de formation de rosettes et d'immunofluorescence. Cette méthodologie a permis notamment de mettre en évidence que la faculté de former des rosettes diminue avec le temps et est pratiquement abolie après 3 à 4 heures de culture avec des IgG anti-erythrocytes. Lorsque la culture est effectuée en présence d'inhibiteurs protéasiques tels que le phenyl methyl sulfonide fluoride (PMSF) même après 4 heures de culture, il est possible d'observer la formation de rosettes.

L'hypothèse du clivage enzymatique est confirmée :

1) Par la démonstration en immunofluorescence de la présence d'un fragment Fc sur la membrane du parasite grâce à l'utilisation d'un $F(ab')^2$ anti-Fc d'IgG ;

2) Par l'apparition et la libération de peptides TCA 6 % solubles dans le milieu de culture ;

3) Par la présence dans le milieu de culture de protéases sécrétées par les larves dont l'une est une sérine protéase neutre, l'autre une metalloaminopeptidase neutre dont les températures optima d'activité sont de 37°C.

Ainsi, après leur fixation sur la membrane des larves, les IgG sont clivées par des protéases sécrétées par le parasite. Ce clivage a pour conséquences de libérer des peptides d'IgG dans le microenvironnement des schitosomules tout en maintenant en place un déterminant du fragment Fc de la molécule.

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Article N 1

Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula

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Summary After the binding of IgG to the surface Fc receptor of *Schistosoma mansoni* schistosomula, the Fab portions of IgG are cleaved and small peptides are liberated in the culture medium. At least two types of proteinase activities have been demonstrated in the secretory products of schistosomula. One is an endoprotease with trypsin-like activity, with an optimum pH of 7 and an optimum temperature of 45°C. The other is a metalloaminopeptidase with an optimum pH of 7 and temperature of 37°C.

Keywords: *Schistosoma mansoni*, IgG, Fc receptor, proteolytic enzymes

Introduction

Preliminary studies have indicated that *Schistosoma mansoni* can acquire host immunoglobulins. It has indeed been shown that host immunoglobulins are associated with the tegumental surface of adult worms (Kemp *et al.* 1977, 1978) and of schistosomula (Torpier, Capron & Ouaissi 1979). These authors have demonstrated by rosette formation the presence on *S. mansoni* schistosomula of a receptor for the Fcγ fragments and for human β_2-microglobulin. Freshly collected schistosomula bind IgG Fc fragments whereas cercariae or 5-day-old lung schistosomula cannot, which suggests the specificity of the early schistosomula stage for immunoglobulin binding. The disappearance of the Fcγ receptor on older schistosomula might result either from the disappearance of the receptor upon maturation or from its blocking by bound IgG on Fc.

In the present work we demonstrate that bound and free IgG both undergo proteolytic cleavage, which produces peptidic fragments liberated in the culture medium. Thus the

Abbreviations LNA = L-leucine-*p*-nitroanilide, MEM = Eagle's minimum essential medium, PBS = phosphate-buffered saline, pH 7·2, BAW = butanol–acetic acid–bidistilled water [4:1:5 (v/v)], BSA = bovine serum albumin, PCMB = parachloromercuribenzoate, PMSF = phenylmethylsulphonide fluoride, TCA = trichloroacetic acid, SIP = schistosomula incubation products.

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Fab fragment, and hence the antibody activity, is lost. A similar proteolysis mechanism has been described in *Tetrahymena pyriformis* (Eisen & Tallan 1977), which is able to cleave IgG combined through their Fab fragments with the parasite surface, and to release univalent antibody fragments.

Both binding and cleavage mechanisms appear as a modulation process which might interfere with the effector or escape mechanisms at the host–parasite interface.

Material and methods

Parasite life cycle and preparation of schistosomula

A Puerto-Rican strain of *Schistosoma mansoni* was used throughout the study (Capron *et al.* 1974). Schistosomula were prepared *in vitro* from cercariae either by a mechanical procedure (Ramalho-Pinto *et al.* 1974) or by the skin penetration method of Clegg & Smithers (1972).

Rosetting assay

Approximately 50 parasites, suspended in 0·1 ml Eagle's minimum essential medium (MEM), were incubated at 37°C for 30 min in an equal volume of anti-sheep erythrocyte rabbit serum (diluted 1:10) or its IgG antibody fraction at a final concentration of 1 mg/ml. After washing three times in PBS, pH 7·2, 2×10^6 washed sheep red blood cells were added in a final volume of 1 ml of MEM. The percentage of rosette-forming parasites was determined by microscopic examination after 30 min of contact at 37°C.

Indirect immunofluorescence test

Fifty parasites suspended in 0·1 ml MEM were incubated for 30 min at 37°C with diluted anti-sheep erythrocyte rabbit serum or the IgG antibody fraction therefrom. After washing three times in PBS, the schistosomula were put in contact for 30 min at 37°C with fluorescein-conjugated $F(ab')_2$ against rabbit IgG (Fc-specific) (FITC–$F(ab')_2$ anti-rabbit IgG Fc; DAKO, Denmark). The parasites were examined after three further washings in PBS under a fluorescence microscope.

Incubation with labelled IgG

Mechanically prepared schistosomula (30 000) were incubated in MEM at 37°C with 50 μg of ^{125}I-labelled (CEA, France) goat IgG (Miles, England), prepared by the chloramine T method as described by Burt & Ada (1969). Two 0·1-ml aliquots were removed after 1, 3, 7 and 16 h of incubation. A control without schistosomula but with 0·1 ml of sterile water added instead in the medium was run in parallel. One millilitre of 1% BSA (Sigma, St Louis, Missouri) was then added to each aliquot, which was precipitated by addition of 1 ml of 20% TCA and filtered through No. 542 Whatman paper for counting in a well-type spectrometer (Intertechnique, Plaisir, France).

Preparation of schistosomula incubation products (SIP)

Mechanically prepared schistosomula were incubated for 4 h in PBS supplemented with 1% glucose to remove the major cercarial enzymes and extensively washed six times. The schistosomula were incubated at 37°C for 16 h in sterile PBS. The absence of bacterial contaminants was controlled.

Substrates

The arylamide substrates (mono- or bi-substituted) were solubilized in methanol (6 mg/ml) and diluted 1:20 in sterile phosphate buffer, 0·2 M, pH 7·2, containing 1:10 000 sodium azide. The synthetic dipeptides were solubilized at 1 mg/ml in the same buffer (all these substrates were kindly donated by Dr Ruffin, INSERM U 16, Unité de Recherche sur les Protéines, whom we wish to acknowledge).

Azocasein (Sigma, St Louis, Missouri) was used at 1% in PBS (containing 1:10 000 sodium azide).

Hydrolysis

With LNA (leucine-*p*-nitroanilide) and other arylamide substrates, 2 ml of substrate and 50 μl of SIP were incubated at 37°C. The yellow coloration indicating the hydrolysis was measured at 410 nm (Beckman DB spectrophotometer). A blank was carried out each time with 50 μl of sterile water without enzymes.

Two millilitres of azocasein and 50 μl of schistosomula incubation products were incubated at 37°C. At the end of hydrolysis, the hydrolysate was precipitated with 2 ml of 20% TCA and filtered through Whatman paper, No. 542. The filtrate was diluted 1:1 in 0·5 N NaOH and left for 1 h at room temperature. The coloration was measured at 440 nm. A blank was carried o t each time using 50 μl of sterile water.

Inhibitors

O-phenanthroline and phenylmethylsulphonide fluoride (PMSF) were solubilized in methanol and used at a final concentration of 1×10^{-3} M. Parachloromercuribenzoate (PCMB) and iodoacetic acid were used at the same concentration in PBS. Soybean trypsin inhibitor (STI) was used at a final concentration of 0·2% and tested with azocasein substrate only, because of the spontaneous degradation of the other substrates.

Gel filtration chromatography

The cleavage products of IgG and intact IgG as a control were separated by gel filtration through ACA 34 Ultrogel (Pharmindustrie, France). The eluate was monitored at 280 nm using an LKB Uvikord II spectrophotometer, and using a ninhydrin reaction on each 2-ml aliquot, according to the method described by Moore & Stein (1954).

Paper chromatography

Unidirectional paper chromatography was used to investigate the hydrolysis of 100 μg

dipeptide substrates by schistosomula incubation products after 4 h at 37°C. The hydrolysates were spotted on No. 3 Whatman paper (46 × 57 cm). Controls of SIP and substrates alone were performed. Elution was carried out for 10 h at 20°C in butanol:acetic acid:water (4:1:5) (BAW). The dried spots were revealed using a 1% ninhydrin solution in ethanol:2-4-6-trimethyl pyridine (19:1).

Results

Disappearance of the Fab fragment 3 h after the binding of IgG

The ability of schistosomula-bound IgG antibodies to combine with their specific antigen in the rosette test indicates that at least some of the immunoglobulins are oriented so as to have one Fab region of the molecule available for binding.

After 3 h of incubation at 37°C (Table 1, Figure 1), IgG antibodies have apparently

Table 1. Rosette formation on schistosomula at different times of incubation in culture medium

	Incubation time in MEM			
Larvae	½ h	1 h	2 h	3 h
Schistosomula*	−	−	−	−
IgG-coated schistosomula†	++++‡	+++	+	(+)

* 2–3-h-old skin schistosomula.

† 2–3-h-old schistosomula were incubated either in rabbit anti-sheep red blood cell diluted serum or an IgG antibody fraction of this same serum at 37°C, washed three times and suspended in 1 ml of culture medium in a Leighton tube.

‡ Each plus sign represents about 20% of rosette-forming parasites (10 or more cells/parasite), (+) = < 20%.

lost their ability to bind to erythrocytes. This suggests either the loss of Fab following a proteolytic cleavage or the shedding of the IgG molecule and/or the Fc receptor.

Small IgG fragments bearing Fc determinants remain, however, on the surface of the schistosomula: using fluorescent $F(ab')_2$ against rabbit IgG (Fc-specific), the IgG-incubated schistosomula are still stained after 3 h of incubation at 37°C (Table 2, Figure 1).

On the other hand, the rosetting by anti-sheep erythrocyte IgG allowed to bind to the schistosomula surface is inhibited 50% by PMSF after 3 h of incubation (but not significantly by *O*-phenanthroline), which supports the hypothesis of an initial protease cleavage destroying the Fab antibody region of the molecule (Table 3).

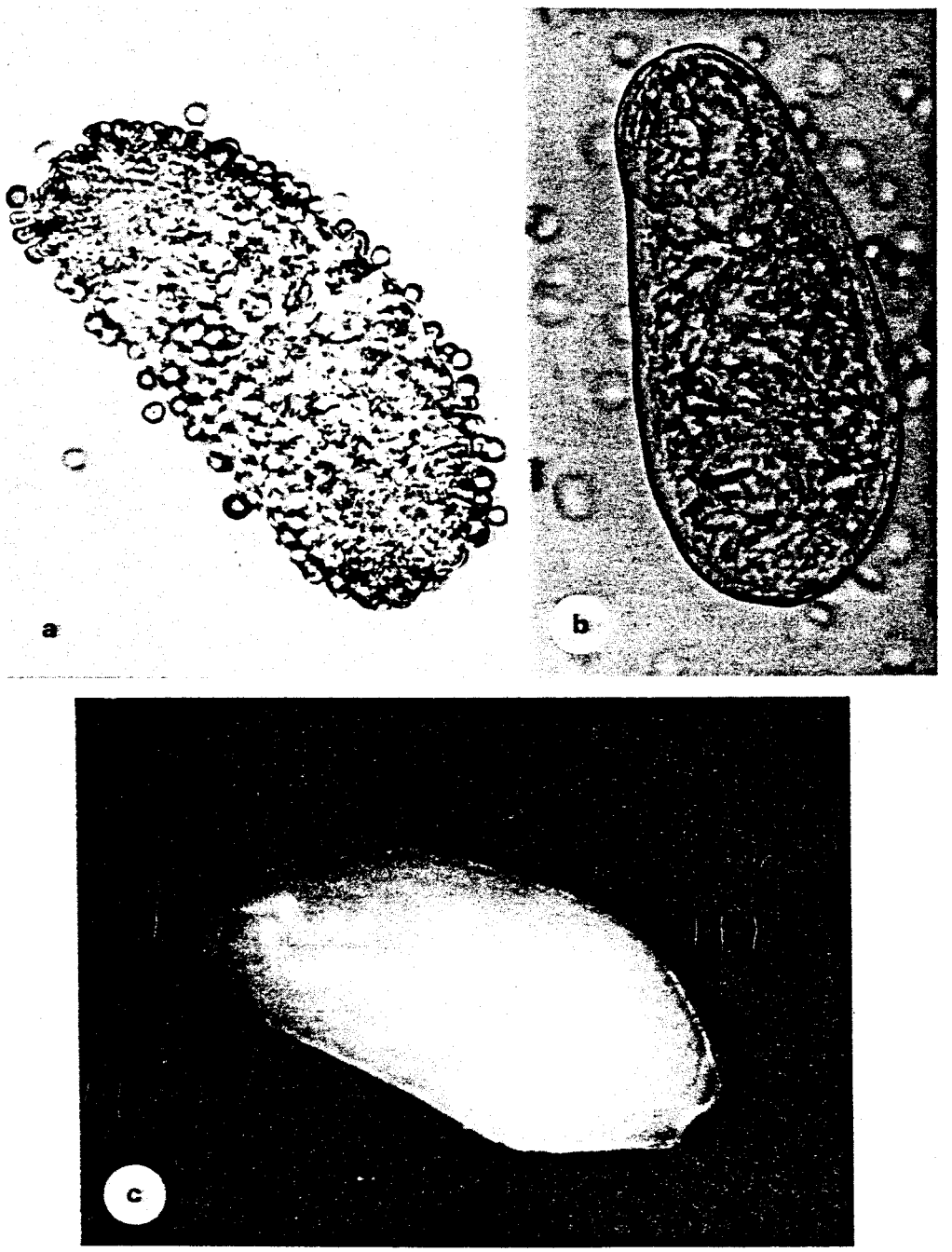

Figure 1. Photomicrographs of rosette formation around schistosomula which had been previously incubated in rabbit anti-sheep red blood cell diluted serum or an IgG antibody fraction of this same serum (**a**). After 3 h of incubation in MEM, IgG antibodies have apparently lost their ability to bind to erythrocytes (**b**). After 3 h of culture in MEM, IgG fragment bearing Fc determinant was revealed by FITC–$F(ab')_2$ anti-rabbit IgG (Fc-specific) on the surface of the schistosomula (**c**). × 700.

Table 2. Binding of IgG to schistosomula at different times of incubation in culture medium, revealed by fluorescence study. The degree of fluorescence was estimated visually and graded from + to +++

	Incubation time in MEM			
Larvae	½ h	1 h	3 h	4 h
Schistosomula	–	–	–	–
IgG-coated schistosomula	+++	++	++	++

Table 3. Effect of PMSF and *O*-phenanthroline on rosette-forming parasites after 3 h of incubation in 1 ml of MEM

	Indicator cells	
Larvae	½ h (%)	4 h (%)
IgG-coated schistosomula +5 µl alcohol	79·6	13·6
IgG-coated schistosomula +PMSF 10^{-4} M	–	39·4
IgG-coated schistosomula +*O*-phenanthroline 10^{-4} M	–	19·6

Cleavage of free and bound labelled IgG

When schistosomula are incubated with ^{125}I-labelled goat IgG, after as early as 1 h, 10% TCA-soluble peptides appear in the incubation medium (MEM) (Figure 2). This shows the ability of the enzymatic material of the schistosomula to cleave IgG into small fragments. A complete hydrolysis is never observed, however, even after a 16-h incubation with the larvae.

When IgG incubated for 16 h with SIP is chromatographed on ACA 34 gel, the majority of cleavage products detected by the ninhydrin reaction are eluted at the end of the filtration. Using appropriate markers, their molecular weight is roughly estimated to be smaller than 12 000 daltons. Neither IgG nor SIP incubated alone and chromatographed as controls show spontaneous degradation (Figure 3).

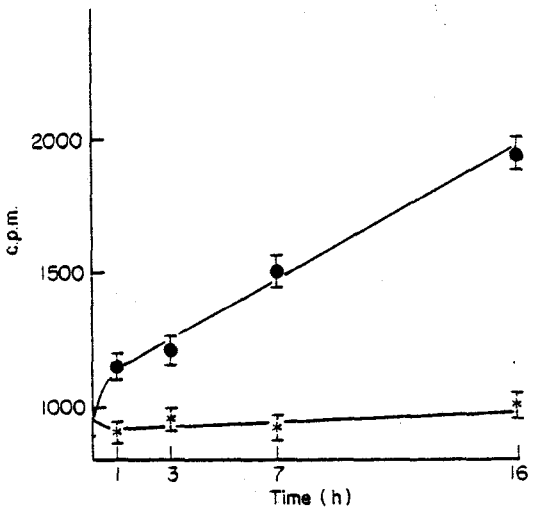

Figure 2. Hydrolysis of goat-labelled IgG (50 μg) incubated at 37°C with (●——●) and without (*——*) schistosomula. Appearance of TCA-soluble peptides in the culture medium (MEM).

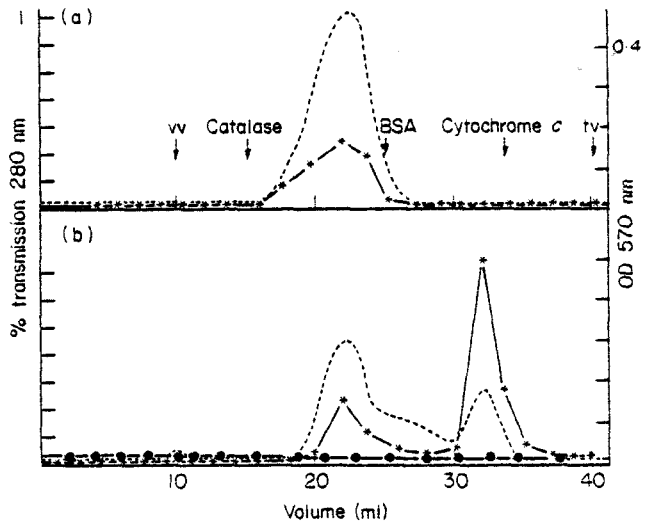

Figure 3. ACA 34 gel filtration of rat IgG (2 mg) (a) and of rat IgG (b), hydrolysed by incubation products of schistosomula for 16 h, monitored as per cent transmission at 280 nm (- - - -) and after ninhydrin reaction of each aliquot at 570 nm (*——*). Controls (●——●) were both carried out after incubation of the incubate only for 16 h. Column: 65 × 1 cm, phosphate buffer, 0·2 M, pH 7·2, void volume 12 ml, fraction volume 2 ml.

Optimum temperature and pH of SIP proteinases

The proteinase activity of SIP is unlikely to cleave IgG molecules exclusively. The hydrolysis of synthetic substrates was thus explored. Since the exposed Fab fragments of an IgG possess NH_2 terminal groups, the presence of an aminopeptidase activity was particularly investigated using LNA substrate. The azocasein substrate was used to study an endoprotease activity.

Optimum temperatures were 37°C for the aminopeptidase activity and 45°C for the protease activity (Figure 4). The optimum pH was determined for both activities as pH 7 (Figure 5).

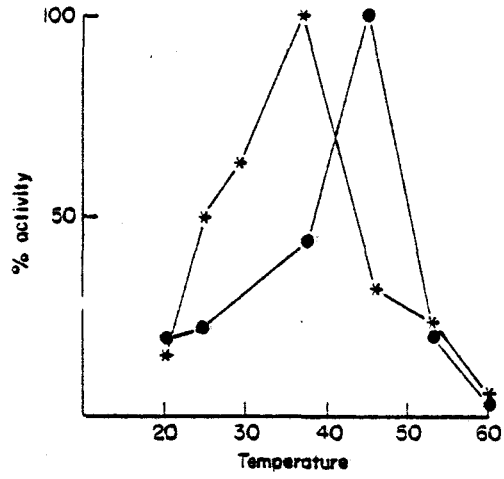

Figure 4. Optimum temperature activity of incubation products of schistosomula using LNA (*——*) and azocasein (●——●) substrates in phosphate buffer, 0·2 M, pH 7·2.

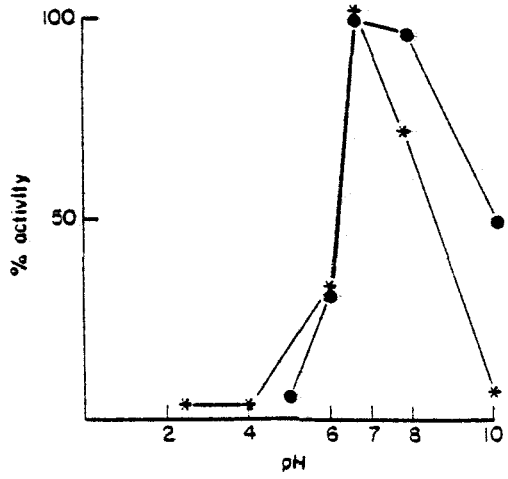

Figure 5. Optimum pH activity of incubation products of schistosomula using LNA (*——*) and azocasein (●——●) in phosphate buffer, citrate buffer and carbonate buffer, 0·2 M, at 37°C.

Effect of enzyme inhibitors on schistosomula proteinases

Results reported above discriminate distinct aminopeptidase and endoprotease activities by their optimum temperature. The effect of enzymatic inhibitors was studied using three substrates—LNA, azocasein and the trypsin substrate benzoil-L-Arg-4NA (Figure 6). As *O*-phenanthroline was shown to inhibit the reaction, the aminopeptidase activity appears to involve at least one metalloaminopeptidase, and the endoprotease activity could be related to one or more serine group, trypsin-like proteases. This hypothesis is corroborated by the effect of soybean trypsin inhibitor, which inactivates 50% of the protease activity on azocasein substrate. On the other hand, 50% PCMB partly inhibits both activities indicating that the active conformation of the enzymatic protein might involve sulphydryl residues.

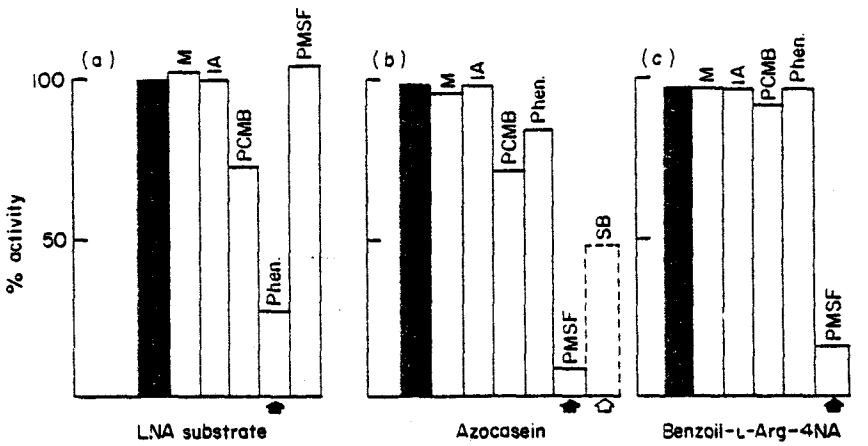

Figure 6. Effect of some inhibitors on the proteinase activity of incubation products of schistosomula using three substrates. (■) Control, M = methanol, IA = iodoacetic acid, PCMB = parachloromercuribenzoate, PMSF = phenylmethylsulphonide fluoride, phen. = *O*-phenanthroline, SB = soybean.

Preliminary studies on the protease specificities of an incubate of schistosomula using synthetic substrates (Table 4, Figure 7)

The identified proteinase activities have different specifities with regard to the hydrolysis of many synthetic peptides. The hydrolysis (4 h) of benzoil-L-Arg-4NA and L-Lys-4NA indicates an endoprotease, trypsin-like activity in SIP, by the presence of mono- or

Table 4. Incubation in phosphate buffer, 0·2 M, pH 7·2, at 37°C of some synthetic substrates with an incubation product of schistosomula

BI-SUBSTITUTED SUBSTRATES	
Acetyl-L-leucine-4NA	−
Glutaryl-L-Phe-L-Ala-4NA	−
Benzoil-L-Arg-4NA	+
MONO-SUBSTITUTED SUBSTRATES	
L-Tyr-4NA	−
L-Pro-4NA	−
L-Lys-4NA	+
L-Ala-4NA	+
L-Leu-4NA (LNA)	+
Gly-L-Phe-L-Ala-4NA	−

+ = Hydrolysed.
− = Not hydrolysed.

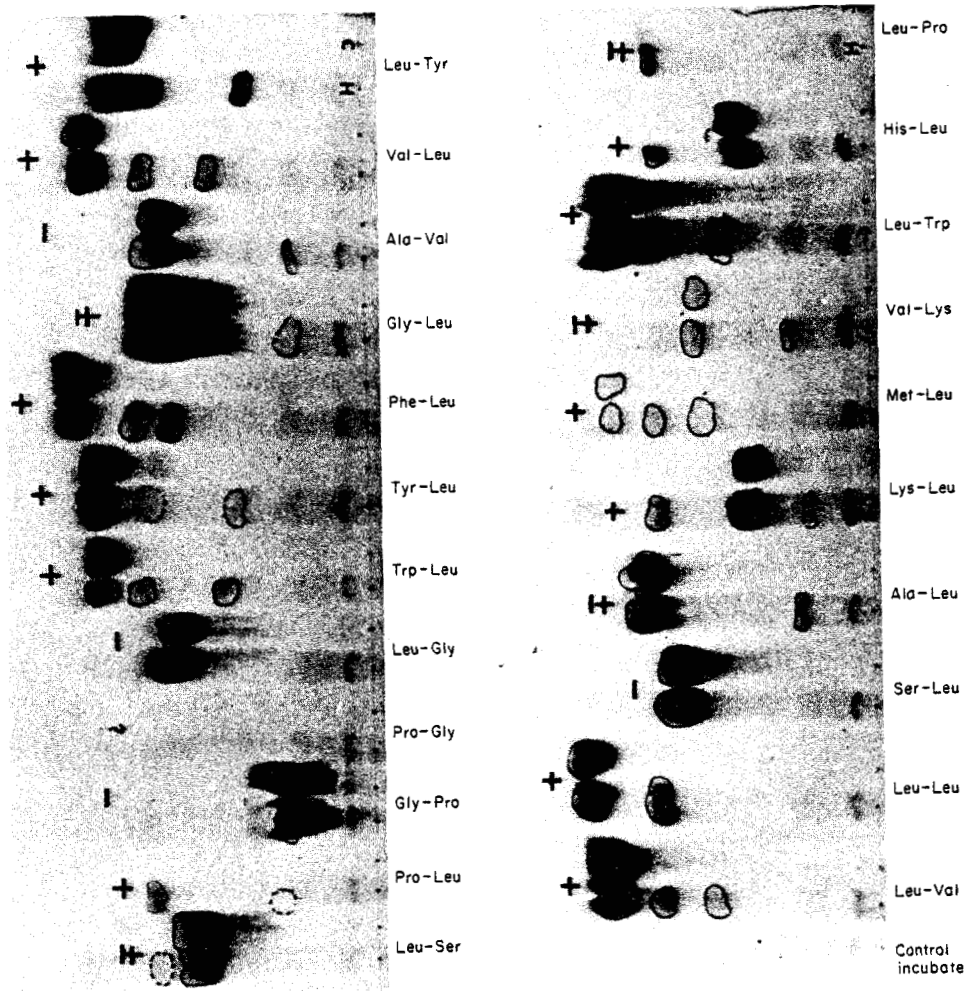

Figure 7. Hydrolysis of some non-substituted synthetic dipeptides by incubation products of schistosomula, followed by a paper chromatography of the hydrolysate in the BAW solvent system revealed after drying by 1% ninhydrin. C = control substrate, H = hydrolysate, + = hydrolysed, − = not hydrolysed, ± = weakly hydrolysed.

bi-substituted basic residues. On the other hand, glutaryl-L-Phe-L-Ala-4NA, Gly-L-Phe-L-Ala-4NA and L-Tyr-4NA which are chymotrypsin substrates are not degraded. Since Leu-4NA (LNA) is hydrolysed, this experiment suggests that the NH_2 group has to be free to allow the hydrolysis of the leucine-*p*-nitroanilide peptide. This confirms the hypothesis of an aminopeptidase activity which is not only a leucine-aminopeptiase since L-Ala-4NA and other non-substituted dipeptides are also hydrolysed.

Discussion

This work had two main objectives:

1 The identification of the demonstration of the cleavage of IgG which is bound to the schistosomulum surface receptor.
2 The identification of the enzymatic materials of schistosomula which perform this cleavage.

The results reported above indicate that the binding of IgG through its Fc piece to the Fc receptor is followed by the cleavage of the molecule into small peptides.

.The presence of at least two proteinase activities in the incubation product of schistosomula suggests that IgG molecules are initially cleaved into large fragments by one or more endoproteases. These fragments are then rapidly hydrolysed in our experimental conditions into small peptides by both aminopeptidases and endoproteases.

The wide specificity of the enzymatic schistosomula materials could explain such a mechanism. But if the proteinase activities are lower in the SIP than in a cercarial extract, they also seem to have different specificities since the cercarial activity has been described previously as a chymotrypsin-like activity (Gazzinelli *et al.* 1966, 1972) (corroborated in our laboratory, unpublished results). This enzymatic switch indicates that the enzymes of the pre-acetabular glands of cercariae do not represent the major protease activity involved in the cleavage of the IgG molecules by schistosomula, and indicates that this phenomenon is a characteristic of the schistosomula stage.

On the other hand, we have demonstrated that many unsubstituted dipeptides with leucine residues in the NH_2 or COOH positions are hydrolysed, even the Leu-Pro and the Pro-Leu dipeptides which are rarely destroyed. This suggests a wide spectrum of the proteinase activities studied. Furthermore, the Tyr-Leu, Phe-Leu and Trp-Leu peptides are hydrolysed in spite of the presence of aromatic residues, while L-Tyr-4NA is not. The previous results exclude a chymotrypsin-like activity which would hydrolyse both the proteins and the mono- or bi-substituted synthetic substrates. Neither endoprotease nor aminopeptidase activities of schistosomula are able to hydrolyse synthetic substrates which possess one aromatic residue. Perhaps the presence of a carboxypeptidase could explain such results.

Finally, some of the peptidic fragments or enzymes themselves could interfere with the immune response. Indeed, some cellular effector mechanisms have already been described in the literature, involving small peptides or proteinases which potentiate cell activity (Ulrich 1979, Najjar & Nishioka 1970, Higuchi, Ishida & Hayashi 1979, Goetzl 1976, Dessaint, Katz & Waksman 1980). The effect of the schistosomula proteinases and of the peptides released from IgG by these proteinases upon effector cells cytotoxic for the larvae is presently being investigated.

Acknowledgements

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Note added in proof: During the preparation of this paper we demonstrated that macrophage activity is inhibited by IgG peptides resulting from cleavage by schistosoma larvae proteases (Auriault *et al.* 1980. *Immunology Letters* **2**, 135).

ARTICLE n° 2 : INACTIVATION DES MACROPHAGES DE RAT PAR DES PEPTIDES RESULTANT DU CLIVAGE DES IgG PAR DES PROTEASES DES LARVES DE S. MANSONI.

ARTICLE n° 3 : INTERACTION ENTRE MACROPHAGES ET S. MANSONI. ROLE DES PEPTIDES D'IgG ET DES AGGREGATS SUR LA MODULATION DE LA LIBERATION DE β-GLUCURONIDASE ET LA CYTOTOXICITE DIRIGEE CONTRE LES SCHISTOSOMULES.

Dans ces deux articles nous mettons en évidence que les peptides libérés après le clivage des IgG par les protéases sécrétées par les schistosomules exercent une action inhibitrice de l'activité des macrophages. En effet la cytotoxicité IgE-dépendante contre les schistosomules par les macrophages est très fortement inhibée (50 à 70 %) lorsque les cellules sont incubées en présence de ces peptides. Par contre, lorsque ces mêmes IgG sont aggrégées, elles exercent au contraire, une action stimulatrice de l'activité macrophagique, rendant même des macrophages normaux capables de tuer les larves, même en l'absence d'anticorps.

Ce mécanisme de régulation faisant intervenir les produits de dégradation et les complexes immuns de la molécule d'IgG, bien que démontré in vitro, pourrait représenter un mécanisme plus général de la régulation des macrophages.

Outre l'inhibition de l'activité anti-schistosome des macrophages, les peptides résultant de l'hydrolyse des IgG par les protéases parasitaires inhibent plus généralement l'activité phagocytaire des macrophages mesurée par les inhibitions :

- de la libération de l'enzyme lysosomiale, la β-glucuronidase ;
- de l'incorporation de glucosamine ;
- de la phagocytose de particules de latex.

Les peptides d'IgG exercent donc un effet inhibiteur non spécifique sans que la viabilité des cellules soit affectée comme en témoignent les contrôles effectués. Par contre, ces peptides ne semblent pas affecter la production d'interleukine-1 par les macrophages, dans les conditions dans lesquelles ils ont été utilisés.

L'électrophorèse sur gel de polyacrylamide du matériel sécrété par la base suivie d'une hydrolyse avec les substrats appropriés, confirme la présence de deux activités enzymatiques majeures dont l'une est une endoprotéase capable d'hydrolyser un substrat comme l'azocaséine. La seconde migrant différemment sur le gel est une aminopeptidase mise en évidence par l'hydrolyse de la leucine p-nitroanilide.

Il est intéressant de noter qu'un hydrolysat de β 2-microglobuline dont Torpier et coll. (1979) avaient montré qu'elle est un compétiteur sur l'accepteur des IgG sur la membrane des schistosomules n'exerce aucun effet sur l'activité macrophagique. Ceci suggère que le peptide inhibiteur n'est pas situé dans les régions homologues des deux molécules, au niveau du troisième domaine constant de la chaine lourde des IgG.

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Article N 2

INACTIVATION OF RAT MACROPHAGES BY PEPTIDES RESULTING FROM CLEAVAGE OF IgG BY SHISTOSOMA LARVAE PROTEASES

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1. Summary

We have previously reported that IgG molecules bound to the surface Fc receptors of *S. mansoni* schistosomula were hydrolyzed by parasite enzymes. In this paper, it is shown that the hydrolyzed peptides inhibit macrophage stimulation, assessed by β-glucuronidase release or glucosamine incorporation, and also reduce both phagocytosis of latex beads and IgE-mediated macrophage cytotoxicity against schistosomula.

This original process might represent an efficient immunosuppressive mechanism of the parasite to escape the host response.

2. Introduction

Previous studies have shown the presence on *Schistosoma mansoni* schistosomula of a receptor for the Fc fragment of IgG and for human β_2 microglobulin [1]. Bound and free IgG both undergo proteolytic cleavage, leading to the release in the culture medium of peptidic fragments, by the action of at least two parasite proteinases: one has been characterized as a neutral serine protease with trypsin-like activity, the other as a metalloaminopeptidase [2].

The purpose of the present study was to investigate the activity of peptides from IgG hydrolyzed with parasite proteases on the activity of peritoneal macrophages.

Abbreviations: SRPH, hydrolysate of IgG by schistosomula released products; SRP, schistosomula-released products without IgG; MDP, muramyl dipeptide; HW, Hank's balanced salt solution; MEM, Eagle's minimum medium.

3. Materials and methods

3.1. *Parasite life-cycle and preparation of schistosomula*

A Puerto Rican strain of *Schistosoma mansoni* was used throughout the study [3]. Schistosomula were obtained from cercariae in vitro by a mechanical procedure previously described [4].

3.2. *Preparation of IgG hydrolysate (SRP) and schistosomula incubation products (SRPH)*

Mechanically prepared schistosomula were preincubated 4 h at 37°C in Eagle's minimal medium (MEM) and extensively washed 6 times to remove most of the cercarial enzymes. The schistosomula (20,000) were incubated for 16 h at 37°C in MEM either with 60 μg/ml of rat IgG (SRPH) or without IgG (SRP). The absence of bacterial contamination was controlled. After elimination of schistosomula by centrifugation, the hydrolysis was stopped by heating for 5 min at 100°C.

3.3. *Macrophage stimulation*

Fischer or Wistar rat resident peritoneal macrophages were purified by the removal of non-adherent cells at the end of a 2-h culture and further incubated overnight in MEM with 10% normal heat-inactivated rat serum. Stimulation by IgE was carried out according to a procedure previously described by Dessaint et al. [5], by a 30 min first-incubation with 10 μg/ml of purified myeloma IgE protein in phenol red-free Hank's balanced salt solution (HW) followed by a second incubation with anti-IgE. Macrophage stimulation by N-acetyl-muramyl-L-alanyl-D-isoglutamine

(MDP) (kindly donated by Prof. Chedid) was induced with 100 ng MDP/ml of HW, for 3 h at 37°C in a 5% CO_2–95% air mixture.

Elicited macrophages were obtained by intraperitoneal injection of 9 ml of a 10% (w/v) proteose–peptone solution (DIFCO) in physiological saline, 4 days before harvesting the peritoneal cells.

SRPH and SRP supernatants were respectively added at a concentration of 50 μl in 1 ml medium/2×10^6 cells to investigate macrophage function.

3.4. *Metabolic and functional parameters*

The extra-cellular release of the lysosomal β-glucuronidase, induced in IgE-stimulated cells by a further 30 min incubation with anti-IgE, was measured according to the procedure of Szasz [6]. The phagocytic activity of resident macrophages was estimated by the uptake of latex beads labeled with tritiated thyramin, as described by Ito et al. [7], using 25 μl of a 10% suspension.

Superoxide anion O_2^- generation was quantified by the cytochrome c reduction test, according to the procedure of Johnston et al. [8] as described by Joseph et al. [9], using IgE-stimulated cells, triggered for 120 min, by anti-IgE-opsonized zymosan in HW.

Tritiated glucosamine incorporation was measured in MDP-stimulated cells by the method of Hammond and Dvorak [10].

Specific IgE-dependent macrophage cytotoxicity against schistosomula was performed as previously described [11] by a 6-h preincubation of the effector cells with *S. mansoni*-immune rat serum.

4. Results

4.1. *Inhibition of macrophage stimulation*

The selective release of lysosomal β-glucuronidase and the incorporation of labeled glucosamine were chosen as two parameters of macrophage stimulation. Table 1 shows that IgG, hydrolyzed by parasite proteases (SRPH) induces a 62% inhibition of the β-glucuronidase release obtained by the IgE–anti-IgE stimulation.

When muramyl-dipeptide was used to trigger the cells, a 70% inhibition of glucosamine incorporation was observed in the presence of hydrolyzed IgG. At the end of each experiment, the surviving macrophages were counted by the trypan blue exclusion test: in all cases more than 90% living cells were observed in the presence or absence of SRPH. IgG hydrolysate as well as SRP were not toxic to the cells and the observed effect was thus related to the inhibition of macrophage activity. Moreover a 24-h culture of normal macrophages in the presence of hydrolyzed IgG resulted in a reduced β-glucuronidase level when compared to the controls, without any modification of the intracellular content in enzymes.

4.2. *Inhibition of phagocytosis*

As shown in Table 3, phagocytosis of [^{3}H]thyr-

Table 1
Intracellular level and 30-min β-glucuronidase-release from IgE–anti-IgE stimulated rat macrophages, with and without IgG hydrolysate (SRPH) (mean ± S.D. of quadruplicate experiments)[a]

First incubation	Second incubation	Release[a]	Intracellular[a]
IgE	anti-IgE	49 ± 2.5[c]	1255 ± 53
IgE + SRPH	anti-IgE	25 ± 2	1127 ± 40
IgE + SRP	anti-IgE	38 ± 4.5	1185 ± 104
IgE + heated SRP[b]	anti-IgE	35 ± 3	1235 ± 109
IgE + SRPH	HW	15 ± 1	ND
HW	HW	12 ± 3	1170 ± 99
HW + SRPH	anti-IgE	10 ± 1	ND
HW + SRPH	HW	19 ± 4	1223 ± 40

[a]Results expressed as nmol substrate/2×10^6 cells/ml.
[b]100°C for 5 min.
[c]Significantly higher than hydrolysate-containing medium ($P = 0.0002$).

Table 2
[^{3}H]-labelled glucosamine incorporation by muranyl-dipeptide-stimulated rat peritoneal macrophages, with and without IgG hydrolysate (mean ± S.D. of quadruplicate experiments)

6-h incubation of 2×10^6 macrophages with	3-h tritiated glucosamine incorporation (cpm)
MDP	2437 ± 279[a]
MDP + SRPH	1538 ± 307
Medium	1119 ± 22

[a]Significantly higher than hydrolysate-containing medium ($P = 0.03$).

Table 3
Effect of IgG hydrolyzed with parasite proteases on phagocytosis of radiolabelled latex beads by rat peritoneal macrophages (mean ± S.D. of quadruplicate experiments)

Macrophages (2.6×10^6)	Phagocytosis (cpm)[a]
incubated alone	32,480 ± 604[b]
incubated 30 min with 50 μl of SRPH	5698 ± 716
incubated 30 min with 50 μl of SRP	20,120 ± 2130

[a]A control without cells gave a background of 6102 ± 2400 cpm.
[b]Significantly higher than hydrolysate-containing medium ($P = 0.0003$).

amin-labeled latex beads by macrophages was strongly inhibited (80–100%) in the presence of SRPH. We noticed likewise a slight inhibition due to SRP alone.

4.3. *Inhibition of cytotoxicity*

When incubated in immune rat serum, normal peritoneal macrophages were strongly adherent to *S. mansoni* schistosomula. This step was followed by a high mortality of schistosomula. When SRPH was added, a sharp decrease in the lethality was noticed, whereas IgE hydrolyzed by parasite enzymes, SRP or non-hydrolyzed IgG had no significant effect (Table 4).

Macrophages incubated with normal rat serum or foetal calf serum were not induced into cytotoxic effector cells either in the presence or in the absence of SRPH.

4.4. *Inhibition of superoxide anion production*

Owing to the reduction of anti-parasite cytotoxicity by the hydrolysate peptides, the production of the cytocidal superoxide anion was measured. The O_2^- generation was inhibited by the hydrolysate, either during IgE, anti-IgE stimulation (100% inhibition) or in macrophages in vivo elicited by proteose peptone (Table 5).

5. Discussion

Cellular effector mechanisms have already been described in the literature, which involve small peptides [12] and particularly IgG peptides [13,14], but all potentiate cell activity. The more precisely

Table 4
IgE-dependent macrophage cytotoxicity against *Schistosoma mansoni* larvae with and without IgG hydrolysate[a]

Normal rat macrophages incubated with					
Immune serum	Immune serum + IgG hydrolysate (SRPH)	Immune serum + IgE hydrolysate	Immune serum + non-hydrolysed IgG[b]	Immune serum + SRP	Foetal calf serum
92.25 ± 1.75	22.5 ± 2.15[c]	74.5 ± 7.80	87.5 ± 0.7	74 ± 11.3	0

[a]Cytotoxicity expressed as % of dead schistosomula after 24 h incubation.
[b]IgG was incubated for 16 h in MEM medium without schistosomula.
[c]Significantly lower than the other conditions with immune serum ($P < 0.0001$).

Table 5
Superoxide anion production by IgE–anti-IgE stimulated, protease–peptone elicited rat peritoneal macrophage with and without IgG hydrolysate (SRPH) estimated by cytochrome c reduction

First incubation	Second incubation	Superoxide anion (nmol reduced cytochrome c (10^7 cells))
HW	anti-IgE-opsonized zymosan	0
IgE + SRPH	NRS-opsonized zymosan	3.8 ± 1.8
IgE + HW	anti-IgE-opsonized zymosan	14.15 ± 0.5[a]
IgE + SRPH	anti-IgE-opsonized zymosan	3.8 ± 2
In vivo activation with proteose peptone		
normal Mϕ + HW		0.45
normal Mϕ + SRPH		5.5
activated Mϕ + HW		9.75
activated Mϕ + SRPH		0.42

[a]Significantly higher than hydrolysate-containing medium ($P = 0.0014$).

described of these peptides is the Tufsin, a tetrapeptide released from enzymatic cleavage of IgG by a neutrophil neutral protease, which stimulates the macrophage and neutrophil phagocytosis.

In the present work, we demonstrated that peptides released from IgG by parasite proteinases strongly inhibited and even abolished various macrophage activities, in stimulated cells. However, products released from schistosomula (SRP), although they had a small inhibitory effect, did not represent the majority of the observed inhibition. But, their combined action with IgG peptides (and even entire IgG) cannot be excluded. The involved active peptide(s) exerted a non-specific inhibitory role since macrophage activity decreased regardless of which stimulation procedure was used. The peptide(s) apparently decreased the stimulation process itself without affecting the basal cell activity when cells were activated or elicited. In our experimental conditions this effect cannot be related to any direct toxic process on the cells since appropriate controls clearly showed that macrophage viability was not affected.

Macrophages being one of the main effector cells involved in anti-schistosomula immunity, their inhibition by peptides derived from a host molecule, bound to a receptor on the larval surface, appears as an essential feature of the host–parasite relationship. Indeed, both the activation and mainly the in vitro anti-schistosomula cytotoxicity are inhibited, producing evidence about the efficiency of this mechanism.

Now, it would be interesting to know whether schistosomula isolated from the lungs of infected animals is also capable of generating such inhibitor peptides.

In conclusion, the whole process could be described in 3 steps: (a) binding of host IgG to the schistosomulum receptor; (b) cleavage of the IgG by the secreted parasite proteases; and (c) maintenance of the effector macrophages in a quiescent stage, thus inhibiting their cytotoxicity by one or more peptides produced by the cleavage of bound IgG.

Though the effects of peptides produced after hydrolysis of other seric proteins bound to schistosomula, in this mechanism, have now to be determined, the results reported here might thus be considered as a new immunosuppressive mechanism, by which the parasite might escape the host immune-response.

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Article N 3

Interaction between Macrophages and *Schistosoma mansoni* Schistosomula: Role of IgG Peptides and Aggregates on the Modulation of β-Glucuronidase Release and the Cytotoxicity against Schistosomula

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Discharge of lysosomal enzymes, measured by release of β-glucuronidase and cytotoxicity against *Schistosoma mansoni* schistosomula, was studied when rat macrophages were incubated in the presence of either IgG peptides, resulting from the cleavage of nonimmune IgG by parasitic proteases, or nonimmune aggregated IgG. With peptides, the macrophage activity showed a dramatic decrease while they were stimulated by IgG aggregates. In contrast, the synthesis of lymphocyte activating factor by macrophages was unaffected. The hydrolysis of IgG is carried out by two distinct enzymatic molecules released into the medium by the larvae. The mechanism by which nonimmune IgG peptides or aggregates inhibit or stimulate macrophage activity, regulated by both parameters indicated above, is discussed and is suggested as a general regulation mechanism for the macrophage activity required for parasite survival in the host.

INTRODUCTION

We have previously reported that IgG molecules, bound to the surface Fc receptor of *Schistosoma mansoni* schistosomula, undergo a proteolytic cleavage (1). At least two types of proteinase activities have been demonstrated in the secretory products of schistosomula which are able to hydrolyze IgG and other synthetic substrates. The first is a neutral endoprotease of the serine group with trypsin-like specificity. The other is a neutral metalloaminopeptidase. This mechanism of binding and cleavage of the IgG molecule by the secreted parasite proteinases generates peptidic fragments which are liberated into the culture medium and strongly inhibit various macrophages functions (2). Indeed, it was shown that the activity of stimulated macrophages, assessed by the release of lysosomal enzymes, labeled glucosamine incorporation, superoxide anion production, or phagocytosis of labeled latex beads, was dramatically decreased when the cells were incubated with IgG peptides before stimulation.

Furthermore, because the macrophage activated by IgE is one of the main types

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of effector cells involved in anti-schistosome immunity with eosinophils, its ability to kill schistosomula in the presence of IgG peptides was tested, and a decrease in the IgE-dependent cytotoxicity was found. These observations indicate that nonspecific IgG could be used by the parasite to modulate the host-immune response from the very beginning of infection.

B-Lymphocyte (3, 4) and human monocyte (5) activities can be modulated by Fc fragments of nonspecific IgG. On the other hand, we have previously shown the induction of β-glucuronidase release from peritoneal rat macrophages by immune complexes (6). The purpose of the present study was to show the role of nonspecific IgG in the activity of macrophages in our parasitic model.

MATERIALS AND METHODS

A Puerto Rican strain of *S. mansoni* schistosomula was used throughout this study (7). Schistosomula were prepared *in vitro* from cercariae either by a mechanical procedure (8) or by the skin penetration method of Clegg and Smithers (9).

Preparation of Aggregated IgG

Heat-aggregated IgG (A-IgG) and crosslinked IgG oligomers: Total rat IgG (500 μl of a 10 mg/ml solution in PBS, pH 7.2) was heat-aggregated at 63°C for 20 min as described by Knutson *et al.* (10). In some experiments rat IgG was crosslinked in the presence of a 30-fold molar excess of dimethylsuberimidate as described for IgE by Segal and Hurwitz (11). IgG oligomers of defined size were obtained after fractionation on an Ultrogel AcA 34 column (IBF, Clichy, France). Before the experiments A-IgG or crosslinked IgG oligomers were dialyzed against culture medium and centrifuged to remove minute amounts of insoluble material (5000*g* for 15 min).

Preparation of IgG Hydrolysate (SRPH) and Schistosomula Incubation Products (SRP)

Mechanically prepared schistosomula were preincubated at 37°C for 4 hr in Eagle's minimum essential medium (MEM) and extensively washed six times to remove most of the cercarial enzymes. Larvae (20,000) were then usually incubated at 37°C for 16 hr in MEM either with 60 μg/ml of rat or human IgG (SRPH) or without IgG (SRP). In preliminary experiments on inhibition of macrophage activity by peptides, SRPH was produced after various times of hydrolysis of IgG by schistosomula (45 min, 1 hr 30 min, and 3, 6, and 16 hr). The hydrolysis was stopped after removal of schistosomula by centrifugation and by heating the supernatant for 5 min at 100°C. The preparations were routinely checked for bacterial contamination.

Incubation with Labeled IgG

^{125}I-Labeled (CEA, France) IgG (Miles, England) prepared by the chloramine-T procedure as described by Burt and Ada (12) was incubated in MEM with mechanically prepared schistosomula. Aliquots of 0.1 ml were removed after 1, 3, 7, and 16 hr of incubation. A control without schistosomula was run in parallel to

verify the spontaneous degradation of IgG. The reaction was stopped by precipitation with 0.6 ml of 20% TCA after addition of 1 ml of 1% BSA, and the mixture filtered through No. 542 Whatman paper for counting in a well-type spectrometer.

Macrophage Stimulation

Wistar rat resident peritoneal macrophages were purified by the removal of nonadherent cells at the end of a 2-hr culture. The cells were then incubated overnight in MEM with 10% normal heat-inactivated rat serum. The macrophage stimulation was carried out first by a 30-min incubation with 10μg/ml of purified myeloma IgE protein in phenol red-free Hanks' balanced salt solution and then by a second incubation with anti-IgE according to the procedure described by Capron *et al.* (13). SRPH and SRP were usually added at a concentration of 50 μl in 1 ml medium per 2×10^6 cells, 30 min before IgE–anti-IgE activation.

Enzyme Assays

(a) The release and the intracellular level of β-glucuronidase, induced in cells stimulated by IgE–anti-IgE were measured according to a modification (14) of the procedure of Szasz (15), using *p*-nitrophenyl-β-glucuronide in acetate buffer as a substrate. After a 4-hr incubation at 37°C nitrophenol absorbance was measured at 405 nm in a Cecil CE 393 spectrometer (Cambridge, England).

(b) In order to estimate cell viability leucine aminopeptidase (EC 3.4.1.1.1), a cytoplasmic enzyme, was measured in the medium as previously described (14), using L-leucine-β-naphthylamide in 0.1 *M* phosphate buffer, pH 7.2, as substrate. After revealing hydrolyzed naphthylamine with fast garnet, GBC absorbance was monitored at 520 nm.

Polyacrylamide Gel Electrophoresis and Enzyme Characterization

Secreted parasitic proteases were characterized using analytical 10% polyacrylamide gel electrophoresis without SDS. The migration was followed with bromophenol blue. Three strips of gel were prepared. One was stained with Coomassie blue. Both unstained strips were cut into slices (2 mm) and placed in appropriate substrates for hydrolysis.

Hydrolysis

(a) For assessment of aminopeptidase activity leucine-*p*-nitroanilide (Sigma, St. Louis, Mo.) was solubilized in methanol (6 mg/ml) and diluted 1:20 in sterile 0.2 *M* phosphate buffer, pH 7.2, containing 1:10,000 sodium azide. Protease activity was assessed using azocasein (Sigma) at a concentration of 1% in sterile phosphate buffer (0.2 *M*, pH 7.2) containing 1:10,000 sodium azide.

(b) Gel slices were incubated in 2 ml of each substrate for 16 hr at 37°C. Coloration resulting from the hydrolysis of leucine-*p*-nitroanilide was measured directly at 410 nm (Beckman DB spectrophotometer). With azocasein, hydrolysates were precipited with 1.6 ml of 20% TCA and filtered through Watman paper No. 542. The filtrate was diluted 1:1 in 0.5 *N* NaOH and hydrolysis was measured at 440 nm. With both substrates a blank consisting of gel slices without parasitic material was carried out at each point.

Cytotoxicity

(a) Specific IgE-dependent macrophage cytotoxicity against schistosomula was brought about as previously described (13) by a 6-hr preincubation of the effector cells with 42-day *S. mansoni* immune rat serum.

(b) For nonspecific cytotoxicity, the macrophage monolayer (250,000 cells in each well), purified as described above, was first incubated with either heat-aggregated IgG or crosslinked IgG oligomers (50 μg/ml) in the presence of 2% guinea pig serum (v/v) as a source of complement. After at least 3 hr about 50-skin-filtered schistosomula were added into each well. After 18 hr of incubation at 37°C the killed larvae were counted.

Lymphocyte Stimulation with LAF

(a) T-Lymphocyte activating factor (LAF) was prepared *in vivo* as in previous studies (16, 17) by a 24-hr incubation of washed peritoneal exudate macrophages elicited by intraperitoneal injection of 9 ml of 10% (w/v) protease peptone solution (Difco) in physiological saline. The supernatant was cleared by centrifugation, dialyzed 24 hr at 4°C against 50 vol of RPMI, and sterilized by filtration.

(b) Macrophage-depleted lymph node cells (LNC) of 2- to 3-month Fisher rats of both sexes were prepared as previously described (17). Cultures were run in triplicate in flat-bottomed microculture plates (Nunclon, Roskilde, Denmark). Each well contained 5×10^5 LNC in a total volume of 0.8 ml of serum-free RPMI, with or without a suboptimal dose of PHA (0.25 μg/ml), LAF (25% v/v), or IgG—either native or hydrolyzed by parasitic proteases (SRPH). Incubation was carried out at 37°C in 5% CO_2 in air. The cells were pulsed with 1 μCi [^{3}H]TdR at 38 hr and harvested at 48 hr on glass fiber filters (Whatman, Clifton, N.J.). The filters were counted for ^{3}H in an ISOCAP 300 spectrometer (Chicago, Ill.).

RESULTS

Inhibition of IgE–Anti-IgE-Induced Glucuronidase Release from Rat Peritoneal Macrophages by Peptides of Nonspecific Rat and Human IgG Hydrolyzed by Parasitic Proteases

The inhibition of exocytosis as measured by β-glucuronidase release was assessed in macrophage monolayers incubated first for 30 min with IgE in serum-free Hanks' balanced salt solution and then with anti-IgE, with or without IgG hydrolyzed by schistosomula proteases. The effect of peptides was investigated according to the time course of IgG hydrolysis by schistosomula proteases (Fig. 1). The inhibition of β-glucuronidase release (and intracellular β-glucuronidase level) increases simultaneously with the generation of 6% TCA-soluble peptides in the culture medium from 45 min to 16 hr of hydrolysis by schistosomula proteases while non-hydrolyzed IgG or parasitic material incubated alone (Figs. 1–3) has no significant effect at the maximum time of hydrolysis. On the other hand, after chromatography of the 16-hr rat IgG hydrolysate through a Sephadex G-25 column (Fig. 2) only the peak corresponding to the low-molecular-weight material (less than 12,000 daltons) inhibits the β-glucuronidase release—evidence of the involvement of peptides in the inhibition mechanism. Hydrolyzed human IgG is also able to strongly inhibit β-glucuronidase release from rat macrophages (80%). Moreover pepsin-

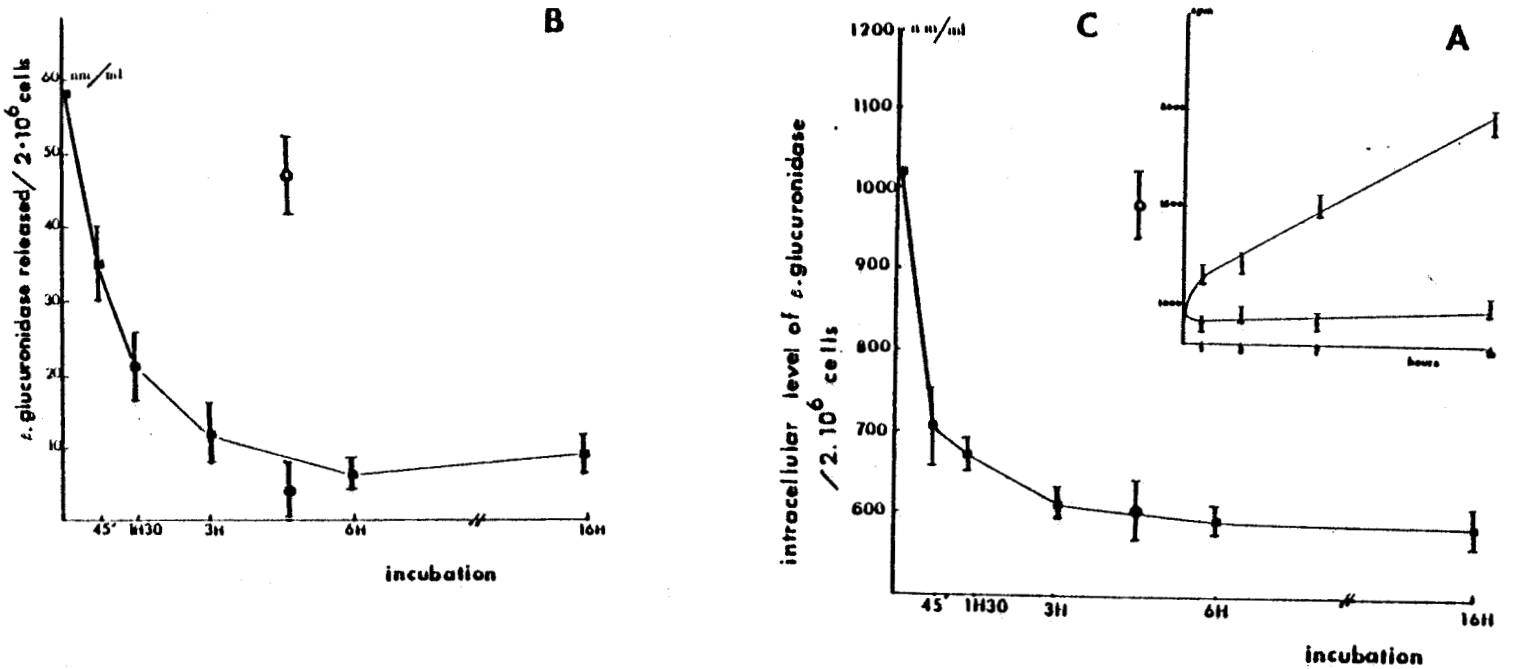

FIG. 1. Relationship between the time course of hydrolysis of IgG by schistosomula proteases and the inhibition of β-glucuronidase release and intracellular level by rat peritoneal macrophages. Results are the mean ± SD of two different experiments carried out in quadruplicate. (A) Appearance of TCA-soluble peptides after hydrolysis of ^{125}I-labeled IgG (50 μg) incubated at 37°C in the presence or absence of living schistosomula. ● — ●, Schistosomula incubated with IgG; ∘ — ∘, control IgG. (B, C) Release and intracellular level of β-glucuronidase by cells incubated with supernatant of SRPH removed at various intervals of time of hydrolysis. Controls consist of IgG and SRP incubated alone with macrophages. ●, Nonstimulated cells; O, IgG; ■, IgG incubated with schistosomula.

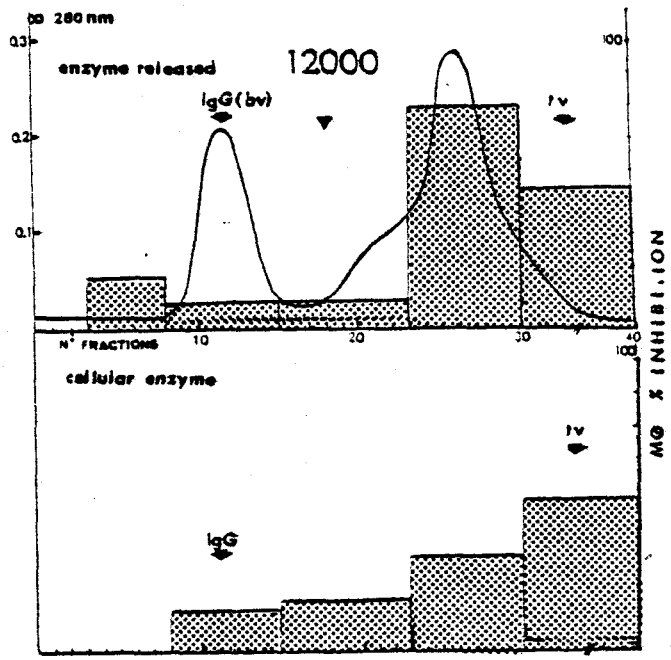

FIG. 2. Inhibition of β-glucuronidase release by rat macrophages incubated with hydrolyzed IgG. Native IgG were incubated for 16 h with enzymatic material of schistosomula filtered through in Sephadex G-25 gel (Column 58 × 1.2 with Hanks' balanced salt solution) and separated into five fractions. These results are representative of two different experiments.

produced $F(ab')_2$ or Fc fragments themselves exhibit a significant inhibitory effect. The rate of inhibition observed after hydrolysis of these fragments by schistosomula proteases was the same as that seen after hydrolysis of the entire IgG molecule, indicating the role of peptide size in this process. On the other hand, entire or hydrolyzed human β-2-microglobulin, which is known to cross-react with IgG on the schistosomula surface (18, 19) does not exhibit a significant inhibitory action on exocytosis.

Activation of IgE–Anti-IgE- and Aggregated IgE-Induced β-Glucuronidase Release from Peritoneal Macrophages by Rat Aggregated IgG

After contact with aggregated IgG, the release of β-glucuronidase into the serum-free medium by rat macrophages prestimulated with IgE and then with anti-IgE antibody or aggregated IgE significantly increases and reaches 49 and 38% respectively, compared with nonstimulated cells (Fig. 4). This increase represents two to three times the β-glucuronidase release of cells stimulated by IgE–anti-IgE or by aggregated IgG alone. This activation is time dependent, since the enzyme release doubles from 3 to 5 hr of incubation.

It can be noted that uncomplexed IgG does not induce a high β-glucuronidase release and therefore cannot be involved in this mechanism of enzyme exocytosis as already mentioned (6). All preparations elicited negligible release of the cytoplasmic marker, LAP.

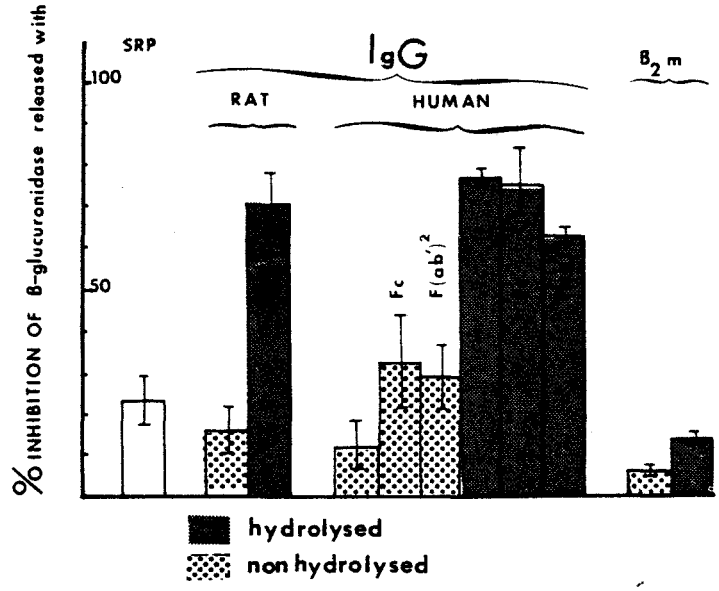

FIG. 3. Inhibition of β-glucuronidase release by rat peritoneal macrophages incubated with: schistosomula-released products alone (SRP); human and rat IgG; human β-2-microglobulin, native or hydrolyzed by schistosomula proteases. Results are the mean ± SD of three different experiments carried out in triplicate.

Effect of IgG Peptides and Aggregates on Anti-Schistosomula Cytotoxicity by Macrophages in Vitro

As for lysosomal enzyme exocytosis, the rate of inhibition of anti-schistosomula cytotoxicity depends on the molecular size of IgG added to the macrophages (Figs.

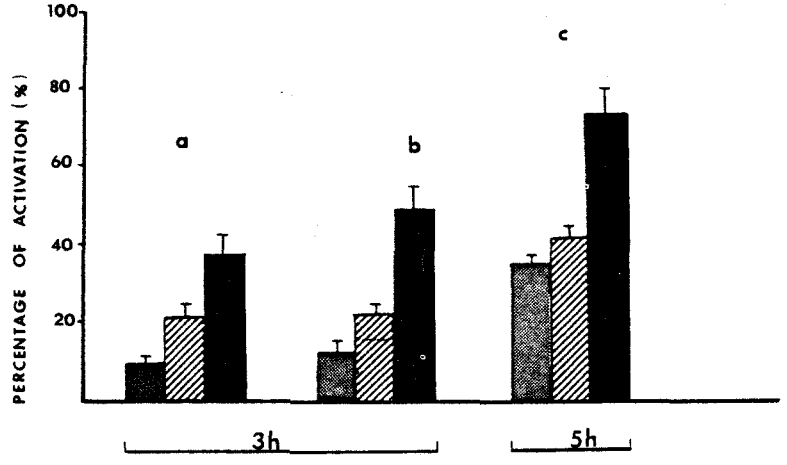

FIG. 4. Demonstration of potential effect of IgG aggregates on β-glucuronidase release by rat peritoneal macrophages stimulated by an IgE–anti-IgE reaction. Macrophage monolayers, ■, were first incubated with IgG aggregates and, after 3 hr, washed and stimulated by IgE aggregates (a), or by IgE–anti-IgE reaction (b); or after 5 hr, washed and stimulated by IgE–anti-IgE reaction (c). Controls were macrophages stimulated by IgG aggregates, ▨, or by IgE aggregates alone and IgE–anti-IgE reaction alone, ▧. Results are the mean ± SD of three experiments in duplicate.

5 and 6). The same batch of nonimmune IgG (Sigma), either hydrolyzed by schistosomula proteases or aggregated with dimethylsuberimidate, was added to the medium with cytotoxic adherent cells and skin-prepared schistosomula. In the presence of IgG dimers or aggregates, the cells become cytotoxic for the larvae (45% of cytotoxicity) without immune serum in the medium but in the presence of at least 2% guinea pig complement (inactive when tested alone). On the other hand, the specific IgE-dependent macrophage cytotoxicity against schistosomula was dramatically decreased (70%) in the presence of IgG peptides whereas IgE hydrolysate, nonhydrolyzed IgG, or parasitic-released material (SRP) had no significant effect.

Parasite Proteolytic Enzymes Responsible for the Presence of Inhibitory IgG Peptides

We have previously reported that endoprotease and aminopeptidase activities are recovered in the incubation medium of schistosomula (1). To determine how many enzymatic molecules were involved in the hydrolysis of IgG we carried out two electrophoresis (− to + and + to −) of the parasitic-released material on a 10% polyacrylamide slab gel (without SDS). The gel was then cut into slices which were

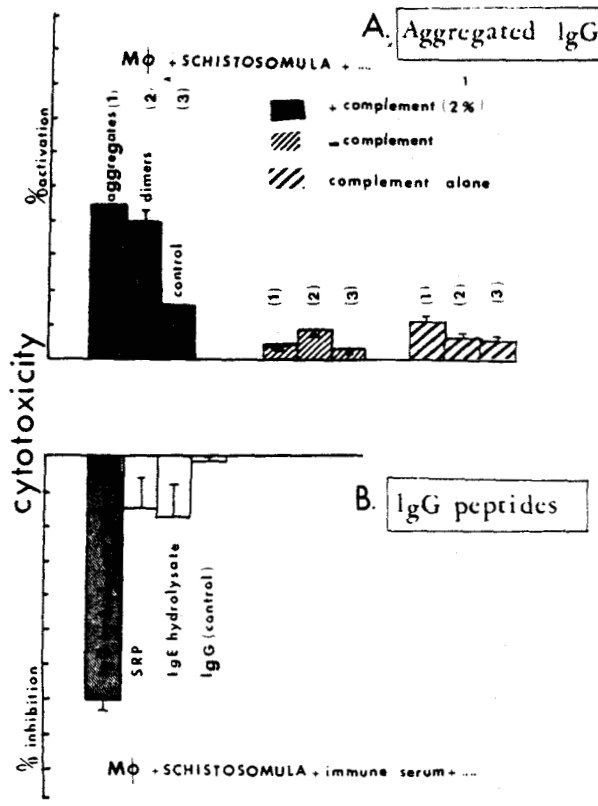

FIG. 5. Macrophage cytotoxicity against *S. mansoni* larvae with normal rat IgG, aggregated with dimethylsuberimidate (A) or hydrolyzed by schistosomula proteases (B). Results are the mean ± SD of three experiments in triplicate.

incubated in a solution of leucine-*p*-nitroanilide, an aminopeptidase substrate, or azocasein, a protease substrate, already used in our previous experiments. The result shows that only one peak of aminopeptidase activity is found in the first half of the gel. Under our experimental conditions this aminopeptidase is well separated from the protease activity found near the origin of the gel (4 mm) in migration − to + exclusively, but not + to − (Fig. 7). More recently we have investigated the activities of these two proteolytic enzymes by other biochemical approaches (manuscript in preparation). Thus, the peptides involved in the inhibitory action of macrophage activity seem to be produced by the combined hydrolysis of the IgG molecules by only two parasitic proteinases: one is a protease able to cleave IgG into large peptides, which are themselves likely reduced into smaller peptides by the aminopeptidase.

Peptides of IgG Hydrolyzed by Schistosomula Proteinases Do Not Inhibit in Vitro Production of Lymphocyte Activating Factor (LAF) from Macrophages

LAF has been described (17) as a macrophage-released soluble factor, the major component of which exhibits a catheptic carboxypeptidase B activity, able to activate lymphocyte metabolism. LAF is recovered in the culture supernatant of macrophages and is tested by addition to a lymphocyte culture. Production of LAF depends directly on macrophage activity and can be used to test the effect of inhibitory peptides. In our experiment we demonstrate that the culture supernatant of macrophages incubated with or without peptides, prepared as described above, has the same effect on the [^{3}H]thymidine incorporation by PHA-stimulated rat lymphocytes (Fig. 8). This indicates that the presence of peptides in the macrophage culture did not modify LAF synthesis and thus did not affect all macrophage functions.

DISCUSSION

In the present study we have extended previous investigations of the interaction of IgG, macrophages, and schistosome larvae by examining the release of β-glucuronidase and the *in vitro* anti-schistosomula cytotoxicity by macrophages. In earlier works we found that peptides, liberated after hydrolysis of IgG and bound onto the schistosomula surface, are able to abolish the phagocytic and cytotoxic activity of macrophages (2). In contrast, normal macrophages, incubated with IgG in the form of either preformed immune complexes or in aggregates, release more β-glucuronidase into the medium than monomeric IgG-stimulated macrophages (24); the results presented here suggest that under our experimental conditions peritoneal macrophage activity can be either activated or inhibited according to the state of the IgG molecules. Indeed, IgG aggregates, even in the dimeric form, are able to stimulate macrophage activity. This indicates that a minimal degree of crosslinking of IgG (either with an antigen or with the same isotype) is necessary to induce a lysosomal enzyme exocytosis or a nonspecific anti-schistosomulum cytotoxicity. Indeed, in the absence of specific antibody, normal *in vitro*-stimulated macrophages become capable of killing the larvae, and this represents a new feature. A small amount of complement is necessary to induce the cytotoxic effect of the macrophages on IgG aggregates, whereas complement alone or IgG-incubated macro-

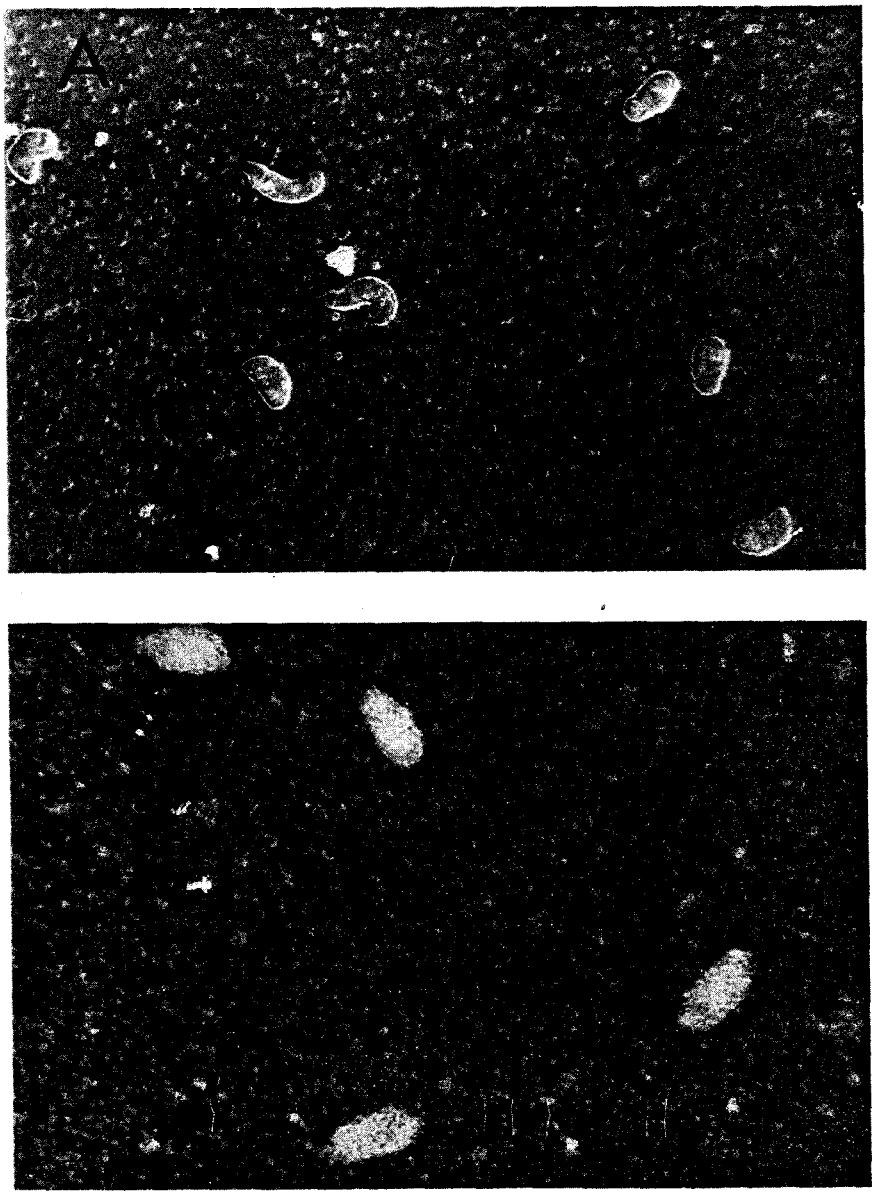

FIG. 6. Microphotographs of antibody-dependent macrophage cytotoxicity against *S. mansoni* schistosomula. Larvae incubated with: (A) normal rat serum-incubated macrophages, (B) 42-day immune serum-activated macrophages, (C) 42-day immune serum-activated macrophages with peptides of protease-hydrolyzed normal IgG.

phages exhibit a negligible action. With the macrophage being a major complement-producing cell on the one hand and IgG or immune complexes possessing a binding sequence on the other hand, both particularities could explain the role of complement in this system. Since complement alone had no effect (at the slight concentration we used) it could act as an amplifier of macrophage activity, by a C_3-

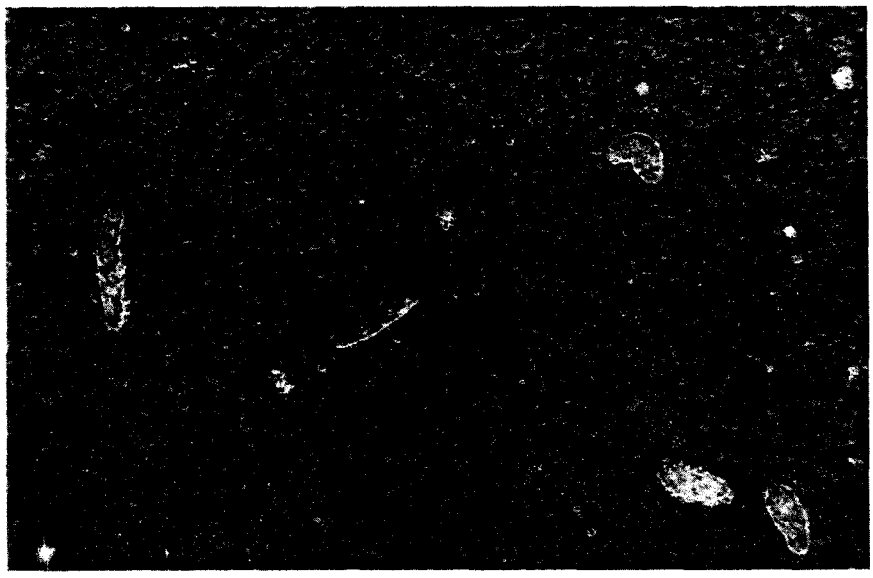

FIG. 6. (*continued*).

mediated adherence as previously described (19) with eosinophils, or could facilitate the fixation of aggregates or immune complexes onto the macrophage and thus increase macrophage-dependent cytotoxicity.

Furthermore, when IgG molecules are shortened by an enzymatic cleavage, the peptides thus produced inhibit the macrophage release of lysosomal enzymes induced by IgE–anti-IgE stimulation and the specific anti-schistosomula cytotoxicity of mac ophages. This points to the role of IgG peptides in a parasitic model since

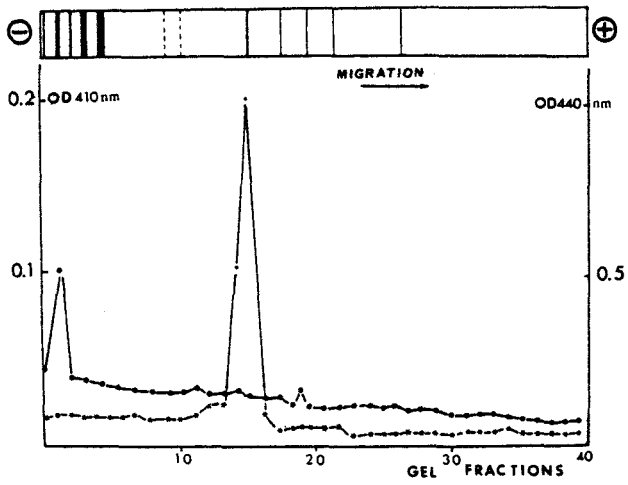

FIG. 7. Characterization of released proteinases from *S. mansoni* schistosomula using analytical 10% polyacrylamide gel electrophoresis. Gels were sliced and then incubated 16 hr at 37°C with (* — *) leucine–*p*-nitroanilide or (● — ●) 1% azocasein both diluted in sterile 0.2 *M* phosphate, pH 7.2.

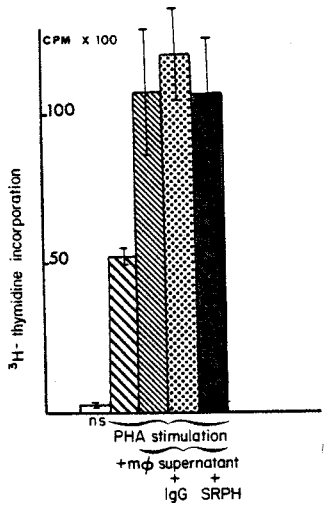

FIG. 8. [^{3}H]Thymidine incorporation by PHA-stimulated rat lymphocytes in the presence of supernatant of macrophages incubated with hydrolyzed (SRPH) or nonhydrolyzed IgG (ns: nonstimulated cells). Results are the mean ± SD of two experiments in triplicate.

it has been established that homologous macrophages incubated with human or rat sera containing IgE antibody to *S. mansoni* become highly cytotoxic for *S. mansoni* schistosomula and accumulate acid hydrolases in their lysosomes (14). Under our experimental conditions, the inhibitory effect of the peptides cannot be related either to any direct toxic process on the cells, since appropriate controls clearly showed that viability was not affected, or to a β-glucuronidase inhibition, since other nonenzymatic parameters were also affected (2). The involved peptides seem to exert a nonspecific inhibitory role but their combined action with parasitic material (SRP) cannot be excluded and must be verified. Because the macrophage is one of the cells involved in defense mechanisms against microorganisms and parasites, its inhibition by peptides could facilitate host invasion by parasites. As soon as IgG is bound, the molecule is rapidly hydrolyzed by the two parasite proteinases described above and liberates macrophage inhibitory peptides into the larval microenvironment. Nevertheless, in contrast with IgG peptides, we have noticed that the hydrolysate of β-2-microglobulin, or peptides of IgE, involved in antibody-dependent cytotoxicity are not able to significantly inhibit macrophage activity.

The functional significance of the peptide and aggregated IgG interaction remains to be determined and the *in vivo* relevance of the macrophage regulation is still under investigation. However, the results presented here, although obtained *in vitro*, could indicate that such a phenomenon is a general mechanism of macrophage regulation used by *S. mansoni* to survive in its host, after penetration and later. Effectively, the release of circulating antigen by adult worms as previously described (20) leads to the formation of circulating immune complexes able, as we have demonstrated using IgG aggregates, to stimulate the macrophage activity and thus to participate in host protection. It is now necessary that we clearly demonstrate IgE–IgG–macrophage interactions and explain the mechanism by which the IgG molecule regulates macrophage functions, especially in terms of the nature and

optimum dose of peptide(s) involved, the possible involvement of Fcγ and ε receptors, and the *in vitro* and *in vivo* action of *S. mansoni* immune complexes.

An interesting analogy can be drawn between the system presently described and the B-lymphocyte regulatory system (3, 4, 21, 22) by the same stimulating or inhibiting agents. But contrariwise, peptides resulting from the cleavage of IgG by macrophage proteases are able to activate the transformation of B cells into secreting cells, whereas the immune complexes exert an inhibitory effect (23). It must be emphasized that B-lymphocyte activator peptides could inhibit macrophage protease release, and consequently peptide production itself, in regulating the immune response.

Precise knowledge of the macrophage activity modulation thus represents an approach to the immunological control of schistosomiasis.

ACKNOWLEDGMENTS

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ARTICLE n° 4 : CARACTERISATION ET SYNTHESE D'UN PEPTIDE INHIBITEUR DES MACROPHAGES SITUE DANS LE SECOND DOMAINE CONSTANT DE LA CHAINE LOURDE DES IMMUNOGLOBULINES G.

Dans cet article, nous montrons que le tripeptide ^{289}Threonyl-Lysyl-Proline^{291} du second domaine constant de l'immunoglobuline G reproduit l'effet inhibiteur de différentes activités macrophagiques des produits d'hydrolyse des IgG par les protéases de schistosomules. Lorsque les macrophages de rats ou humains sont préincubés avec ce tripeptide (500 nanomoles de peptides/ml pendant 30 minutes) préalablement à la réaction IgE-antiIgE, le relarguage de β-glucuronidase est significativement diminué. Les macrophages humains de patients allergiques et stimulés avec l'allergène correspondant (Dermatophagoïdes pteronysinus) par l'intermédiaire du récepteur IgE sont également inhibés ce qui souligne l'intérêt pharmacologique de ce peptide, et la possibilité de son application dans d'autres domaines que les affections parasitaires. En même temps que sa libération, le taux intracellulaire est diminué, ce qui suggère que le peptide intervient au niveau de la régulation de la synthèse de cette enzyme lysosomiale. Le tripeptide Thr-Lys-Pro inhibe également la migration des macrophages de rats ainsi que la génération d'ions superoxydes (O_2^-).

Ce seul peptide donc peut être responsable de l'effet inhibiteur des fonctions macrophagiques dès les premiers stades de l'infection parasitaire. Ceci n'exclut pas que d'autres peptides d'IgG peuvent également intervenir et leur identification est actuellement en cours.

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Article N 4 FEBS Letters

CHARACTERIZATION AND SYNTHESIS OF A MACROPHAGE INHIBITORY PEPTIDE FROM THE SECOND CONSTANT DOMAIN OF HUMAN IMMUNOGLOBULIN G.

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SUMMARY

It was previously shown that IgG hydrolysed by Schistosoma mansoni schistosomula inhibited various macrophage functions, especially phagocytosis and anti-schistosome cytotoxicity. In the present study, we demonstrate that a tripeptide, Thr-Lys-Pro, of the second constant domain of human Immunoglobulin G (peptide 286-292) reproduced the inhibitory effect of a total hydrolysate. Indeed the β-glucuronidase release from IgE-anti-IgE-stimulated rat and human macrophages decreased and its intracellular level did not rise after a prior incubation of the cells with Thr-Lys-Pro(500nmoles/ml). Moreover, the cell migration as well as the superoxide anion O_2^- generation were 50 to 80 % reduced by the tripeptide. These results suggest that a single peptide set may be responsible for the decrease of the macrophage functions at the early stage of the parasite infection in the mammalian host. The pharmacologic properties of this tripeptide are under investigation.

1 - INTRODUCTION

We have previously reported that IgG, bound onto the surface of Schistosoma mansoni schistosomula, was cleaved by proteases secreted by the larvae. This process of binding and cleavage of IgG molecules generates peptidic fragments, liberated in the larval environment, which interfere with various macrophage functions [1]. Indeed, it was initialy shown that a total IgG hydrolysateinhibited the macrophage stimulation assessed by β-glucuronidase release and glucosamine incorporation, and reduced both phagocytosis of latex beads and IgE-mediated macrophage cytotoxicity against schistosomula [2]. The IgG hydrolysate apparently prevented the cell stimulation itself, without affecting its viability. IgE-activated macrophages being one of the main effector mechanisms involved in the anti-schistosome immunity, its inhibition by such peptides could, at least in part, facilitate host invasion by parasites. But more generally, the inhibiting effect on the phagocytic activity of the macrophage suggests the use of the active peptides as molecules with pharmacologic goals.

In the present study we have demonstrated that a tripeptide* from the second constant domain of human IgG reproduced the inhibiting effect of a total IgG hydrolysate on several macrophage functions.

* French Pat. Application N° 82. 12222 filed July 2, 1982.

2 - MATERIAL and METHODS

2.1. Preparation of IgG hydrolysate

Schistosomula of a Puerto-Rican strain of Schistosoma mansoni were obtained from cercariae by a mechanical procedure in vitro previously described [3]. The larvae were incubated for 16 h at 37°C in MEM with 60 µg rat IgG/ml. The absence of bacterial contamination was controlled. After elimination of schistosomula by centrifugation, the hydrolysis was stopped by heating the supernatant 5 min at 100°C.

2.2. Metabolic and functional parameters

The extracellular release of the lysosomal β-glucuronidase, induced in IgE-stimulated cells by a further 30 min incubation with anti-IgE, was measured according to the procedure of Szasz [4].

Superoxide anion O_2^- generation was quantified by the reduction of cytochrome c according to the procedure of Johnston et al. [5] as modified by Joseph et al. [6] with IgE-stimulated cells triggered for 120 min by anti-IgE-opsonized zymosan in phenol-red free Hank's balanced salt solution (HW).

Specific IgE-dependent macrophage cytotoxicity against schistosomula was performed as previously described [7] by a 6 h-preincubation of the effector cells with S. mansoni-immune rat serum.

2.3. Cell viability

In all cases, more than 90 % living cells were observed in the presence or absence of IgG peptides, estimated by the trypan blue exclusion test and the fluorescein diacetate method [8].

2.4. Macrophage triggering by IgE

For human alveolar macrophages, the free lung cells were recovered after informed consent from non-atopic patients, by bronchoalveolar lavage performed under fiberoptic bronchoscopy. The cell pellets of centrifuged lavage fluids, consisting of 93 % alveolar macrophages and 7 % lymphocytes, were resuspended in MEM with 10 % foetal calf serum, and incubated for a 2h-adherence phase. After removal of unadherent cells, the macrophages were 98 % pure, and further incubated overnight in the same medium.

Rat peritoneal macrophages were purified to 95 % by adherence, for 2 h, and incubated overnight like human alveolar macrophages.

Both cell populations were treated at 37°C with IgG peptides (500 nanomoles/ml) for 30 min, washed, and then exposed to IgE triggering [6 ; 9]: a first 30 min-incubation at 37°C in 10 µg IgE/ml HW was followed, after washing, by a second 30 min-period in 10 µl anti-IgE antibody/ml HW. Supernatants were recovered, and cells lysed with Triton X-100 0.1 % in Tris-$MgCL_2$ 0.01 M pH 7.4, for extra and intracellular lysosomal enzyme assay.

2.5. Macrophage chemotaxis

Rat peritoneal macrophages were purified to 95 % by a 2 h-adherence phase, incubated for 30 min with IgG peptides, washed, and recovered by scrapping. The macrophage-enriched population was adjusted to 10^6 cells/ml MEM with 10 % foetal calf serum, and used for chemotaxis. Macrophage migration was assessed at 37°C for 2 h in a 48 well-microchemotaxis assembly (Neuroprobe Corporation, Bethesda, Maryland), with a 5 µm porosity polycarbonate filter (Nuclepore Corporation, Pleasanton, California) [10] and 10 % zymosan activated normal rat serum in MEM as chemoattractant. The filter was stained with Giemsa, and chemotaxis was quantified by counting and averaging the macrophages that migrated completely through the filter, and present on its lower surface.

2.6. PEPTIDES

Peptide synthesis were performed by the solid phase method [11] in an automated Beckman synthesizer Model 990B according to a previously described protocol [12]. N - Boc - amino acids were used and trifunctional amino acids were protected as N - Boc - Arg (Tos), N - Boc - Lys (Z) and N - Boc - Thr (Bal). Asn was incorporated with DCC/HOBT coupling as proposed by Mojsov et al. [13]. The protected peptidyl-resins were cleaved by a 1 hour HF treatment in the presence of anisole. The crude peptides were first purified by gelfiltration (G10, Pharmacia) followed by preparative reversed-phase chromatography (Lobar C_8, Merck). The purified peptides were checked by analytical HPLC, TLC in two different solvant systems and amino-acid analysis of their acid hydrolysates.

3. RESULTS

When the rat macrophages were incubated with various peptides of the second constant domain of human IgG before IgE triggering, a decrease of the selective lysosomal enzyme release (Table I) and of the chemotaxis (Table 2) could be observed. Thr-Lys-Pro exhibited the maximum inhibitory effect. In contrast, the hexapeptide Asn-Ala-Lys-Thr-Lys-Pro was without effect on the macrophage activity, while the penta- and tetrapeptides showed intermediate activity. Furthermore, the tetrapeptide Tuftsin as well as its competitive inhibiting tripeptide Lys-Pro-Arg, had no effect on the triggering by IgE. Thr-Lys-Pro exhibited the same inhibitory effect on the superoxide anion O_2^- generation by rat macrophages, and reproduced the inhibition observed with an hydrolysate of IgG by schistosomulum-secreted proteases (Table 3). This last observation may be related to the reduction of the IgE-dependent cytotoxicity expressed by rat macrophages against schistosome larvae after incubation with the tripeptide : in such conditions, macrophages exhibited a 38 % decrease in the in vitro killing of schistosomula.

Besides its activity on rat macrophages, the same tripeptide proved to be inhibitory also for human alveolar macrophages during the interaction with IgE, either as myeloma protein or as allergen-specific antibody (Table 4). For both rat and human macrophages, in addition to the inhibitory effects on enzyme exocytosis, the intracellular β-glucuronidase, in the presence of the tripeptide Thr-Lys-Pro, remained at the level of unstimulated cells when compared to optimally triggered phagocytes.

4. DISCUSSION

In this work, we have demonstrated that peptides of the second constant domain of human IgG molecules strongly inhibited various macrophage activities during IgE-anti-IgE stimulation and, more particularly, the release of lysosomal enzymes such as β-glucuronidase, and the superoxide anion O_2^- generation. The cell migration was also significantly reduced. The inhibitory effect of the tripeptide Thr-Lys-Pro decreased upon the addition of aminoacids to the NH2 position (Asn 286, Ala287, and Lys 288), or to the COOH position (Arg 292)of the IgG sequence. The peptide apparently interfered with the stimulation process itself, without affecting the basal cell activity. Indeed this effect could not be related to any direct toxic action on the cells, since appropriate controls clearly showed that macrophage viability was not altered. The tetrapeptide Tuftsin (Thr-Lys-Pro-Arg), known to increase the macrophage functions,had neither activating nor inhibiting effect on the rat macrophages when added during the IgE-antiIgE triggering. This could be related to the high efficiency of the IgE-antiIgE reaction to stimulate the macrophage [14] . In addition, Lys-Pro-Arg, which is a competitive inhibitor of Tuftsin [15] , was without effect in the triggering and inhibitory processes described here. Therefore, to interfere with the macrophage activity, Thr-Lys-Pro probably does not exert its action through the Tuftsin receptors of the cells, and may use cellular targets distinct from these reported in the Tuftsin system. Moreover, Thr-Lys-Pro was never described as an inhibitor of Tuftsin.

Of particular interest was the interaction of this peptide with the triggering of human alveolar macrophages by the serum of asthmatic patients with was shown to induce a rapide and strong IgE-dependent stimu-

lation of the cell metabolism and secretory functions upon the addition of specific allergens [6, 16].

Though the effect of this tripeptide on the macrophage activity resembles that described for the whole hydrolysate of IgG by parasitic proteases, it is not excluded that other peptides from the IgG molecule could play such an inhibitory role, and their identification is presently under investigation. Nevertheless, the COOH-terminal proline of the tripeptide suggests the need of prolidase activity among the proteases secreted by the young larval stage of schistosome. Indeed, some of them exhibit prolidase and collagenase specificities [1].

An immunopharmacologic study of this tripeptide and of its analogues is presently in course in our laboratory.

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TABLE I : Release and intracellular [illegible] following incubation with IgG peptides of the second constant domain.

First incubation	Second incubation	N[a]	Enzyme release [b]	% inhibition	Intracellular [b] level
IgE	Anti-IgE	15	12.6 ± 0.9	0	241 ± 13
IgE + Asn-Ala-Lys-Thry-Lys-Pro[c]	Anti-IgE	2	15.6 ± 5.8	-	260 ± 32
IgE + Ala-Lys-Thr-Lys-Pro	Anti-IgE	4	11.1 ± 3.4	15	222 ± 33
IgE + Lys-Thr-Lys-Pro	Anti-IgE	10	10.6 ± 3.1	20	213 ± 27
IgE + Thr-Lys-Pro	Anti-IgE	13	5.4 ± 0.4	73	174 ± 16
IgE + Tuftsin : Thr-Lys-Pro-Arg	Anti-IgE	2	10.6 ± 0.2	20	244 ± 12
IgE + Lys-Pro-Arg	Anti-IgE	5	10.3 ± 0.8	23	247 ± 13
IgE + IgG hydrolysate	Anti-IgE	7	6.9 ± 0.7	58	185 ± 16
HW	HW	15	2.8 ± 0.4	100	204 ± 14

[a] Number of experiments in triplicate

[b] nmoles of hydrolysed substrate/10^6 cells (mean ± s.e.m.)

[c] All peptides were used at the concentration of 500 nmoles/ml

TABLE 2 : Chemotaxis of rat peritoneal macrophages after incubation with peptides of the second constant domain of IgG.

Macrophages pre-incubated with	Number of cells [a]	% inhibition
HW	44.1 ± 8.0	-
HW + Asn-Ala-Lys-Thr-Lys-Pro [b]	50.1 ± 8.9	-
HW + Ala-Lys-Thr-Lys-Pro	27.8 ± 2.7	37
HW + Lys-Thr-Lys-Pro	25.2 ± 4.0	43
HW + Thr-Lys-Pro	6.6 ± 2.7	85

[a] Mean of 16 optic fields of experiments made in triplicate

[b] All peptides were used at the concentration of 500 nmoles/ml

TABLE 3 : Superoxide anion generation by IgE-antiIgE triggered rat peritoneal macrophages in the presence of IgG-peptides.[a]

Preincubation with	Superoxide anion [b]	% inhibition
Medium alone	71.7 ± 10.4	-
IgG hydrolysate	19.3 ± 9.1	73
Tripeptide (Thr-Lys-Pro)[c]	35.4 ± 1.06	51

[a] Triggering signal given by IgE (30 min) followed by anti-IgE opsonized zymosan

[b] nmol of SOD inhibitable reduced chytochrome c/10^7 cells

[c] Used at the concentration of 500 nmoles/ml

TABLE 4 : Release of β-glucuronidase from IgE/anti-IgE and allergen-stimulated human alveolar macrophages after incubation with an IgG tripeptide of the second constant domain.

First incubation	Second incubation	Enzyme release [a]	% inhibition	Intracelular level
IgE	Anti-IgE	87.5 ± 8.0	0	1757.5 ± 6.8
IgE + Thr-Lys-Pro [b]	Anti-IgE	37.5 ± 3.0	71	1692.5 ± 37.0
HW	HW	17.5 ± 3.0	—	1668.2 ± 16.0
Serum from athmatic patient	Allergen [c]	35.0 ± 0.5	0	875.0 ± 5.0
Serum from asthmatic patient + Thr-Lys-Pro	Allergen	22.5 ± 3.5	63	831.5 ± 7.5
HW	HW	15.0 ± 0.5	—	781.5 ± 40.5

[a] Results expressed as nmol substrate/10^6 cells

[b] Peptide used at the concentration of 500 nmoles/ml

[c] Allergen (Dermatophagoides pteronyssinus) used at the concentration of 100 ng/ml HW

ARTICLE n° 5 : INTERACTION ENTRE SCHISTOSOMA MANSONI ET LE SYSTEME DU COMPLEMENT. ROLE DES PEPTIDES DE LA REGION Fc DES IgG DANS L'ACTIVATION DE LA VOIE CLASSIQUE PAR LES SCHISTOSOMULES.

Pour répondre à la question : Quelles sont les conséquences fonctionnelles du clivage des IgG par les protéases parasitaires ? outre la libération des peptides inhibiteurs des macrophages, il restait à définir si le fragment Fc maintenu en place sur la membrane des schistosomules peut avoir une incidence quelconque sur les relations hôte-parasite. Il avait été précédemment mis en évidence par Santoro et coll. (1979) que les schistosomules peuvent activer le complément par les voies alternes et classiques. De plus, il avait été précisé que l'activation de la voie alterne entraine la mort des larves immatures in vitro. L'activation de la voie classique par contre nécessite la présence d'IgG et n'est pas cytolytique. Dans cet article, nous montrons que les schistosomules incubés avec des peptides d'IgG hydrolysés par des protéases sécrétées par les schistosomules peuvent initier l'activation du complément par la voie classique, à en juger par les consommations des facteurs C1, C2 et C4. Une éventuelle activation par des IgG non hydrolysées aggrégées est à exclure puisque aucune consommation de C4 n'est observée dans un sérum agammaglobulinémique restauré en IgG et incubé à 37°C pendant 45 minutes sans schistosomules. D'autre part ces résultats éliminent également l'intervention des protéases parasitaires elle-même puisque lorsque les schistosomules sont incubés avec les parasites de sécrétions seuls (SRP) dans lesquels se trouvent les enzymes, le C4 n'est pas consommé. Ceci est d'ailleurs confirmé par le

fait que le chauffage à 100°C pendant 3 minutes de l'hydrolysat d'IgG avant leur incubation avec les schistosomules n'affecte en rien la consommation de C4. Il existe donc une étroite relation entre les produits de dégradation des IgG et les schistosomules qui a pour conséquence l'activation du complément par la voie classique et qui de plus nécessite la fixation des peptides d'IgG sur la membrane des larves. Les peptides d'IgG peuvent être remplacés par les seuls fragments Fc de la molécule alors que la partie Fab n'entraine pas la consommation de C4 par les schistosomules. De plus, il semble que les peptides d'IgG ou les Fc à la même concentration sont plus efficaces que les immunoglobulines entières puisque des concentrations dix fois moins importantes conduisent aux mêmes résultats.

Une étude par la technique d'immunofluorescence montre que l'activation de la voie classique nécessite la liaison de la partie Fc des IgG et ce fragment de molécule servira lui-même de ligand au premier composant du complément le C1q.

Ceci suggère donc que la partie Fc restée en place à la surface des schistosomules après le clivage des IgG par les protéases parasitaires entraine l'activation du complément par la voie classique. Ce mécanisme en lui-même n'étant pas cytolytique, ceci pourrait avoir pour conséquence d'annuler les mécanismes effecteurs par anticorps spécifiques ou cellulaires non spécifiques dépendant du complément. L'acquisition des antigènes d'hôte d'origine complémentaire comme le C1q et le C3b peut également s'expliquer par l'intermédiaire de ce mécanisme.

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NTERACTION BETWEEN *SCHISTOSOMA MANSONI* AND THE COMPLEMENT SYSTEM: ROLE OF IgG Fc PEPTIDES IN THE ACTIVATION OF THE CLASSICAL PATHWAY BY SCHISTOSOMULA[1]

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It was previously reported that *Schistosoma mansoni* chistosomula in the presence of IgG activate comple- ıent through the classical pathway (CCP). In the present ork we have demonstrated that schistosomula incu- ated with IgG peptides resulting from IgG hydrolysis by chistosomula proteases are able to initiate complement ctivation through CCP, since a marked consumption of :1, C4, and C2 was observed. Our results eliminate an ventual direct action of the substances released by chistosomula on the activation of CCP. The first step of :CP activation required the preliminary binding of IgG peptides on the schistosomula surface. This interaction nduced an increase in the C1q uptake by schistosomula. Since the involvement of the Fc portion of IgG molecules ıas been clearly evidenced, in this case the peripheral globular subunits of C1q recognize the CH_2 region on IgG peptides or intact IgG molecules. By this mechanism, the ocal consumption of complement around schistosomula could contribute to its survival in the host.

Previous *in vitro* studies have established that *Schistosoma mansoni* cercariae activate complement (C) through the alternative pathway (1). Recently, it has been demonstrated (2) that *S. mansoni* schistosomula are able to activate complement by both the classical (CCP)[3] and the alternative pathways (ACP). Moreover, it has been shown that activation of the alternate pathway and late complement components induce killing of these immature schistosomes *in vitro* (2, 3). On the other hand, the activation of the CCP by schistosomula involves the presence of IgG (2).

In a previous study (4) we demonstrated that the Fab portions of IgG bound to the parasite surface were cleaved and small peptides were liberated into the culture medium. However, a small IgG fragment bearing Fc determinant remained on the surface of schistosomula. In addition, we found (5) that binding of C1q to schistosomula can occur directly by specific C1q receptors, and indirectly through the IgG molecules attached to the parasite surface. Only this second mechanism seems to be involved in CCP activation by schistosomula (2). Therefore, in the present study we attempted to investigate the role of schistosomula-bound Fc peptides in the activation of the CCP.

MATERIALS AND METHODS

Parasite cycle and preparation of schistosomula. A Puerto Rican strain of *Schistosoma mansoni* schistosomula was used throughout this work, as described in (6). Schistosomula were prepared artificially from cercariae by the technique of Ramalho-Pinto *et al.* (7), or by the skin penetration method described by Clegg and Smithers (8).

Media and reagents. The culture medium used in these experiments was Eagle's minimum essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 200 IU penicillin/ml and 50 μg/ml streptomycin. Purified human IgG, IgG (Fc), and F(ab)'2 fragments were supplied by Nordic Immunological Laboratories (Tilburg, The Netherlands).

Sera. A pool of normal human serum (NHS) was obtained from normal healthy donors. C4-deficient guinea pig serum was obtained from a colony maintained at the Institut Pasteur in Lille. Blood was allowed to clot at room temperature for 3 hr, and the serum was removed by centrifugation and was used immediately or, when necessary, was stored at −70°C. Human sera with a total deficiency of C1 or C2 and "agammaglobulinemic" human serum (human serum defective in IgG; hsd) containing less than 18.8 IU/ml of IgG were used, as previously described (2).

Complement components. C1q was prepared according to the method of Yonemasu and Stroud (9) as modified by Zubler and Lambert (10).

Direct fluorescence. Fluorescein isothiocyanate (FITC)-labeled C1q (FITC-C1q) was obtained following a method described previously (5). Five aliquots (a, b, c, d, e) of newly prepared schistosomula (800) in 0.1 ml MEM were prepared as follows: a) Schistosomula were incubated with FITC-C1q alone. b) Schistosomula were mixed for 30 min at 37°C with C1q (0.25 μg), washed in MEM, and incubated in the same medium for 1¼ hr. c) Schistosomula were treated with C1q for 30 min at 37°C, washed in MEM, and incubated with IgG peptides (100 μl of SRPH) for 45 min at 37°C. After 2 washes in MEM, the parasites were mixed with C1q for 30 min at 37°C. d) Schistosomula were incubated with IgG peptides for 30 min at 37°C. After 2 washes in MEM, the larvae were then incubated in the same medium for 1¼ hr at 37°C. e) Schistosomula were mixed with C1q for 30 min at 37°C, washed in MEM, and put in contact with IgG peptides for 45 min. After 2 washes in MEM, the larvae were incubated in MEM for 30 min at 37°C. The different aliquots of schistosomula (a, b, c, d) were washed in MEM. The fluorescence test was performed by incubating the individual aliquot with 50 μl of the FITC-C1q solution (1 mg of labeled C1q/ml) diluted ¼ in PBS for 30 min at 37°C. After 3 washes in PBS, the parasites were examined under a fluorescent microscope. Living schistosomula exhibiting surface membrane fluorescence were graded + to ++. Negative schistosomula showed no yellow-green fluorescence around the outer edges. Dead or damaged larvae were not assessed for fluorescence.

Preparation of IgG hydrolysate by parasite enzymes (SRPH) and schistosomula incubation products (SRP). Mechanically prepared schistosomula (20,000) were preincubated at 37°C for 4 hr in Eagle's minimimal medium (MEM) and were washed 6 times to remove most of the cercarial enzymes. The larvae were then incubated for 16 hr at 37°C in 2 ml of MEM either with 40 μg/ml of human IgG (SRPH) or without IgG (SRP). The removal of schistosomula was carried out by centrifugation, and the hydrolysis was stopped by heating the supernatant for 5 min at 100°C. The absence of bacterial contamination was routinely controlled.

Complement consumption assay. This was performed according to Santoro *et al.* (2). Briefly, 800 skin-prepared schistosomula suspended in 0.1 ml of MEM were incubated either with 0.2 ml of normal human serum (NHS) or "agammaglobulinemic" human serum (hsd). In other experiments, different aliquots of schistosomula were incubated at 37°C for 45 min with 1 of the following: 100 μl of a solution of 400 μg/ml of purified human IgG, 100 μl of SRPH containing 4 μg of hydrolyzed human IgG, 100 μl of schistosomula incubation products (SRP), 100 μl of IgG-Fc fragments (400 μg/ml), or 100 μl of F(ab)'2 fragments (400 μg/ml). The larvae were then washed 3 times and incubated with "agammaglobulinemic" human serum. After 45 min incubation at 37°C, the levels of C1, C4, and C2 were measured on hemolytic plates as described by Lachmann and Hobart (11). The results are expressed in percentage of C consumption as compared with the respective levels obtained with 0.2 ml NHS diluted in 0.1 ml MEM.

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[3] *Abbreviations used in this paper:* CCP, classical complement pathway; ACP, alternative complement pathway; FITC, fluorescein isothiocyanate; SRPH, IgG hydrolysate by schistosomula proteases; SRP, schistosomula incubation products; hsd, human serum defective in IgG.

RESULTS

Consumption of CCP conponents during the incubation of human era with Schistosomula. When schistosomula were incubated with ormal human serum (NHS), a high percentage consumption of C4 vas obtained (Fig. 1). In addition, a close correlation was observed etween the number of schistosomula and the percentage consumption of C4. In contrast, there was no consumption of C4 when uman serum defective in IgG (hsd) was used instead of NHS. Iowever, when hsd was reconstituted with IgG, C4 was consumed t a high level (Fig. 1). Moreover, when freshly collected schistosomula were incubated with IgG (40 μg) or IgG hydrolysate (SRPH), vashed, and put in contact with hsd, a high consumption of C1, C4, and C2 was observed (Figs. 2 and 3; Table I). However, while μg of intact IgG were unable to induce C4 consumption by schistosomula, the SRPH obtained after hydrolysis of 4 μg of IgG y schistosomula proteases gave a significant increase of C4 consumption (Table I; Fig. 5). This indicates that proteolyzed IgG t the same concentration as intact IgG (4 μg) was more efficient n inducing CCP activation by schistosomula. The following treatments were done as controls: 1) Schistosomula were incubated vith hsd alone. 2) schistosomula release products (SRP) were dded to hsd without schistosomula and incubated in the same onditions. 3) hsd was reconstituted with IgG and incubated without schistosomula. 4) SRPH alone was added to hsd without chistosomula. In these controls, no consumption of C4 was observed (Fig. 3; Table I). These results strongly support the notion hat activation of CCP by schistosomula requires the presence of ntact IgG molecules or IgG hydrolysate obtained after the hydrolsis of purified human IgG by schistosomula proteases.

Consumption of C4 as a function of the time course of the ydrolysis of IgG by schistosomula proteases. The following assays ere used: In a first step, 80 μg of human IgG were incubated with 0,000 schistosomula in 2 ml of MEM; after 1, 3, 6, and 16 hr of ncubation, 1 aliquot (100 μl) was removed and put in contact with ewly prepared schistosomula (800). After 45 min of incubation at 7°C the larvae were washed 3 times and put in contact with uman serum defective in IgG (hsd). After 45 min incubation at 7°C, the level of C4 was measured. Two milliliters of schistosomula release products (SRP) were incubated with human purified gG (80 μg) at 37°C; different aliquots (100 μl) of IgG hydrolysate SRPH) were then removed at 1, 3, 6, and 16 hr of incubation. In second step newly prepared schistosomula (800) were incubated ith these same aliquots of SRPH for 45 min at 37°C, washed 3 mes in MEM, and put in contact with hsd for 45 min at 37°C. The vel of C4 was then measured. As controls, 80 μg of human IgG 2 ml of MEM without schistosomula, and 2 ml of schistosomula

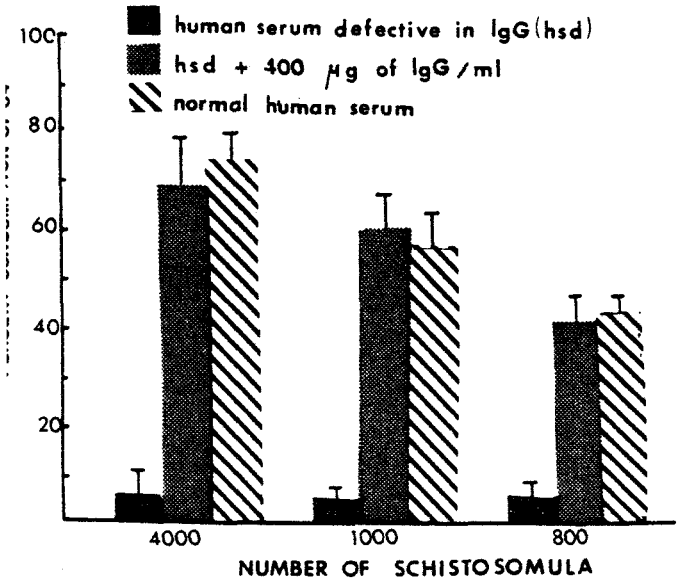

Figure 1. C4 level after the incubation of an "agammaglobulinemic" human rum (hsd) alone or reconstituted with IgG and of the normal human serum with fferent quantities of schistosomula (mean ± SD of 3 experiments). The conmption of C4 required the presence of IgG.

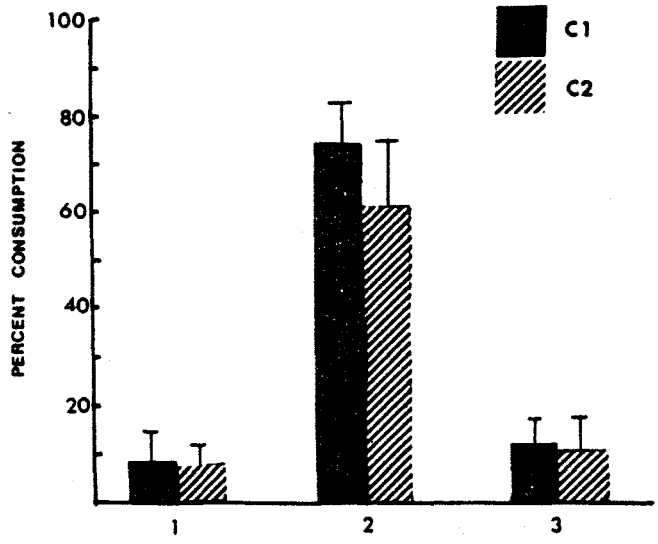

Figure 2. Consumption of C1 and C2 during the incubation of 800 schistosomula with human serum defective in IgG alone (hsd) (*1*) or IgG peptides (SRPH) corresponding to the hydrolysis of 4 μg of IgG followed by a contact with hsd (*2*). As control schistosomula released products (SRP) were added to hsd without schistosomula (*3*). The results represent the mean ± SD of 3 experiments and are expressed in percentage of consumption as compared with the respective levels obtained with the same untreated serum.

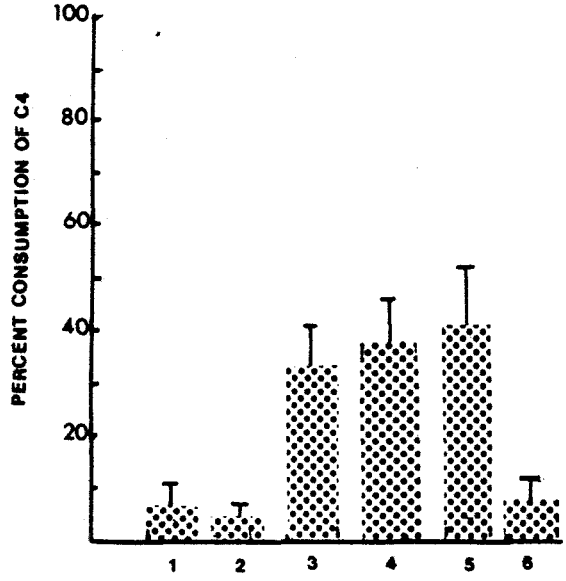

Figure 3. C4 consumption obtained after different treatments of schistosomula. Schistosomula were incubated with human serum defective in IgG alone (hsd) (*1*): schistosomula were treated either with 40 μg of human IgG (*3*) or heated (*4*) or nonheated (*5*) IgG peptides (SRPH) corresponding to the hydrolysis of 4 μg of IgG, and then put in contact with hsd. As controls hsd was mixed with either 40 μg of human IgG without schistosomula (*2*) or schistosomula released products (SRP) (*6*). These results represent the mean ± SD of 3 experiments.

release products (SRP) were incubated in the same conditions. After 1, 3, 6, and 16 hr, aliquots of 100 μl were removed and put in contact with newly prepared schistosomula, and then treated in the same conditions.

As shown in Figure 4, no consumption of C4 was observed in the controls. In contrast, when schistosomula were previously treated with SRPH, the consumption of C4 was related to the kinetics of IgG hydrolysis. In fact, a close relationship was observed between the period of hydrolysis and the percent consumption of C4. These results demonstrate that at a low concentration (4 μg), IgG alone was inefficient in inducing C4 consumption by schistosomula. In contrast, after the hydrolysis by schistosomula proteases, IgG at a low concentration became able to mediate C4 consumption.

Involvement of IgG Fc peptides in CCP activation by schistosomula. These experiments were done in order to determine the

TABLE I

C4 consumption by schistosomula mediated by IgG peptides

	Treatments		% C4 Consumption (Mean ± SD of 3 Experiments)
	45 min	45 min	
In the presence of 800 schistosomula	Intact IgG (40 μg)	hsd	38 ± 4.5
	Intact IgG (4 μg)	hsd	7 ± 1
	SRP[a]	hsd	7 ± 3
	SRPH[b]	hsd	51 ± 11
Controls without schistosomula	Intact IgG (4 μg)	hsd	4 ± 1
	SRP[a]	hsd	4.5 ± 1.5
	SRPH[b]	hsd	5.5 ± 2.5

[a] Schistosomula-released products.

[b] Peptides resulting from the hydrolysis of 4 μg of IgG (see *Materials and Methods*).

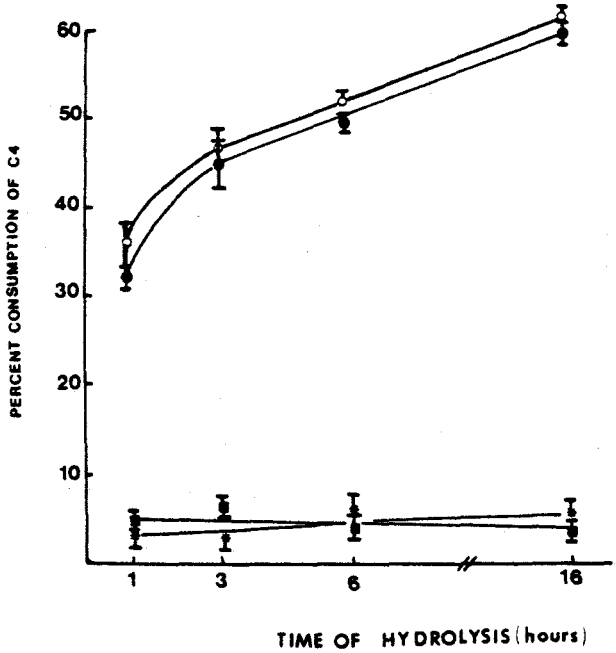

Figure 4. Relationship between the time course of the hydrolysis of human IgG by schistosomula proteases and the consumption of C4. Human IgG (80 μg) were incubated with 20,000 schistosomula in 2 ml of MEM (●——●) or with 2 ml of schistosomula (20,000) released products (SRP) (○——○). After 1, 3, 6, and 16 hr of incubation at 37°C one aliquot (100 μl), corresponding to 4 μg of hydrolyzed IgG, was removed and then incubated during 45 min at 37°C with newly prepared schistosomula (800). The larvae were then washed and put in contact with hsd. As controls, aliquots of 100 μl of intact human IgG corresponding to 4 μg of IgG incubated at 37°C but without schistosomula (■——■) or 100 μl of SRP (*——*) were removed after 1, 3, 6, and 16 hr of incubation and tested in the same conditions. These results represent the mean ± SD of 3 experiments.

respective roles of the IgG peptides in the activation of CCP by schistosomula. Because of the difficulty in separating IgG peptides from SRPH, which, in addition, contains schistosomula release products, these studies were performed with purified Fc and Fab fragments of human IgG. The following treatments were done: The schistosomula (800) in 100 μl of MEM were incubated with either purified Fc or Fab fragments (400 μg/ml) of human IgG. After 45 min at 37°C, the larvae were washed 3 times in MEM and, in a second step, were incubated with 200 μl of human serum defective in IgG (hsd). After 45 min at 37°C the level of C4 was measured as described above. In these experimental conditions consumption of C4 was only observed with the larvae previously treated with Fc fragments of human IgG (Fig. 5). No consumption was observed with schistosomula incubated with hsd alone or previously treated with Fab fragments of human IgG before contact with hsd. These results clearly indicated the direct involvement of Fc fragment of human IgG in the activation of CCP by schistosomula.

FITC-C1q binding to IgG-peptide-coated schistosomula. We have previously reported that IgG molecules bound to the surface of *S. mansoni* schistosomula were hydrolyzed by parasite enzymes (4). However, a small IgG fragment bearing Fc determinants remains on the surface of schistosomula. In addition, 2 possible means of C1q binding to schistosomula have been demonstrated: 1) by a specific C1q receptor, and 2) through the IgG previously attached by its Fc fragment to the parasite surface (5). Only the second mechanism seems to be involved in CCP activation by schistosomula. However, we had not explored the exact mechanism of C1q interaction with IgG molecules. We have attempted by the following complementary investigations to see whether IgG peptides instead of intact IgG molecules can mediate the binding of C1q to schistosomula. The incubation of schistosomula with purified C1q completely inhibited subsequent FITC-C1q binding on their surface (Table II[c]). However, the fluorescence was still present when the larvae were mixed with IgG peptides after the incubation with C1q (Table II[f]). This positive fluorescence was inhibited by a second treatment of the larvae with purified intact C1q (Table II[d]). Treatment of schistosomula with IgG peptides before the contact with FITC-C1q gave an increase in the positive fluorescence (Table II[e]). These results indicated that C1q can bind to schistosomula by both its specific receptor and by the IgG peptides attached to the parasite surface.

DISCUSSION

Previous *in vitro* studies have established that *S. mansoni* schistosomula are able to activate complement (C) in the absence of antibodies, by both the classical (CCP) and the alternative pathways (ACP). The authors clearly demonstrated that C activation via the AP induced killing of these immature schistosomes *in vitro* (2,

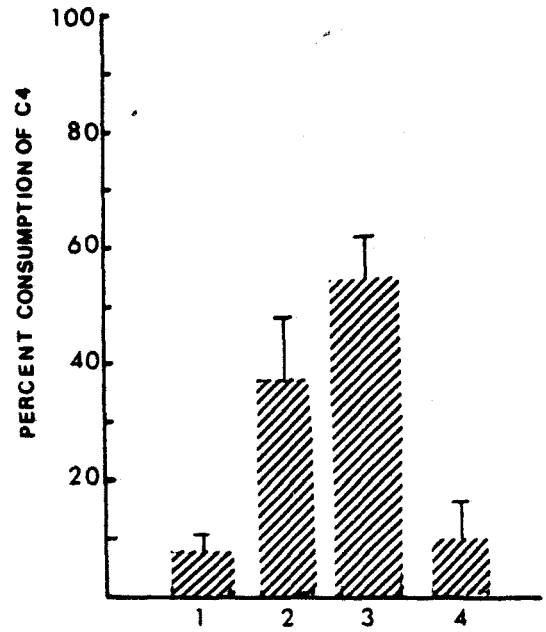

Figure 5. C4 consumption by 800 schistosomula previously treated with either purified Fc (40 μg) (3) or Fab fragments (40 μg) (4) of human IgG before the incubation with hsd. As controls, schistosomula were incubated with hsd alone (1) or hsd supplemented with human IgG (2) (40 μg). The consumption of C4 was only noticed in the presence of IgG (2) or IgG-Fc fragments (3). These results represent the mean ± SD of 3 experiments.

TABLE II

Binding of human FITC-C1q to schistosomula mediated by IgG peptides (SRPH) released after cleavage of IgG molecules by schistosomula proteases

Treatments				Fluorescence[a]
30 min	45 min	30 min	30 min	
—[b]	—	—	FITC-C1q	+
C1q[c]	—	—	FITC-C1q	−
C1q[d]	IgG peptides	C1q	FITC-C1q	−
IgG peptides[e]	—	—	FITC-C1q	++
C1q[f]	IgG peptides	—	FITC-C1q	+

[a] The degree of fluorescence was estimated visually and graded from + to ++.

[b–f] See *Materials and Methods.*

However, it has been shown that activation of the CCP is not fficient for the killing unless specific antibodies to the schistomula were present (2, 3, 6). Unlike oncornaviruses, which could tivate CCP through C1 receptors present on their surface (12,), the CCP activation by schistosomula was dependent on the esence of IgG, since no consumption of C1 or C2 or C4 was ıserved when schistosomula were incubated with "agammaglobnemic" human serum alone. However, the CCP components ere normally consumed with the same "agammaglobulinemic" ıman serum reconstituted with IgG. The exact mechanism of CCP tivation is unclear. The purpose of the present study was to plain the mechanism and the incidence of this phenomenon in e host-parasite relationship.

Our results corroborate the findings above, since the CCP activation by schistosomula was related to the presence of IgG. In ddition, by using IgG peptides resulting from the previous cleavge of IgG molecules by proteases secreted by the larvae (SRPH) stead of intact IgG molecules, it was demonstrated that schistomula can activate the CCP. In fact, no C1, C4, or C2 consumption as found with the "agammaglobulinemic" human serum unless lis same serum was reconstituted with IgG hydrolysate (SRPH). ot schistosomula alone or SRPH or SRP tested alone were able) initiate the CCP. In fact, at a low concentration the hydrolysis of gG significantly increased the C4 consumption, suggesting that it ; likely that the binding of IgG peptides on the larvae is required or the activation of the CCP by schistosomula.

We have previously reported that IgG molecules undergo a first roteolytic cleavage soon after binding to the schistosomula membrane, liberating the Fab portions of IgG molecules in the medium 4). A small IgG fragment bearing Fc determinants remains on the chistosomula surface. The small peptides resulting from this hyrolysis exert a significant inhibition of macrophage activity. They vere especially able to decrease dramatically the *in vitro* IgE-lependent macrophage cytotoxicity against *S. mansoni* schistomula (14, 15). The role of the remaining IgG-Fc portion had to e determined. We have demonstrated in the present study that nly IgG-Fc fragments were involved in the CCP activation by chistosomula. In fact, no C4 consumption was observed when chistosomula were incubated with F(ab)'2 fragments of human gG before the mixture with "agammaglobulinemic" human serum. n contrast, a great increase of C4 consumption was noticed with gG-Fc fragments treated with schistosomula. In addition, the binding of FITC-C1q on the schistosomula depends in part on the resence of IgG peptides in the medium. Since the Fab fragments of IgG cannot bind to the schistosomula surface (16), the involvement of the Fc peptides could be in a bridging by their CH_2 domain between the larvae and the peripheral globular subunits of C1q molecules. This interaction could be the first step of the CCP activation by schistosomula.

Activation of complement by parasites was found to be lethal in most of the experiments that were performed *in vitro* (17); however, in some cases it could be essential for the development of parasitemia (18, 19). In schistosomiasis, it has been shown that the activation of the ACP was lytic for most of the schistosomula that had been incubated with fresh normal serum (2). However, the activity of normal sera against schistosomula was variable according to the types of animal sera and schistosomula used. On the other hand, when normal human serum was used, it was shown that the ACP was not efficient against skin schistosomula. In the present study, all the experiments of complement consumption by schistosomula were performed using skin schistosomula and normal or IgG-deficient human sera. In these conditions, the CCP activation did not induce killing of schistosomula *in vitro*. Since C3 is the common pivotal molecule in both classical and alternative pathways, schistosomula C3 receptors (20) could function in binding to and inactivating C3b as it is generated. Such a blockage could inhibit the feedback cycle and a proliferative C3b-9 complex formation. This hypothesis appears more tenable when viewed in the context of recent work of Fearon (21) and Nussenzweig and Kyoko (22). Thus, it should be emphasized that local consumption of complement around schistosomula could be one of the mechanisms that contribute to its survival in the host.

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ARTICLE n° 6 : ACTIVATION DES EOSINOPHILES DE RAT ET HUMAINS PAR DES FACTEURS SOLUBLES LIBERES PAR LES SCHISTOSOMULES DE S. MANSONI.

ARTICLE n° 7 : ACTIVATION DES FONCTIONS EFFECTRICES DES EOSINOPHILES PAR DES FACTEURS SOLUBLES LIBERES PAR S. MANSONI : ROLE DES PROTEASES.

Lorsque nous avons voulu tester l'effet d'un hydrolysat d'IgG par des protéases parasitaires sur l'activité cytotoxique des éosinophiles de rats vis-à-vis des schistosomules, nous avons constaté :

1) que les peptides d'IgG n'inhibaient pas la fonction effectrice de ces cellules ;

2) que les produits de sécrétion des larves avaient au contraire un effet stimulant sur leur activité à en juger par l'augmentation de la cytotoxicité dépendant des IgG2a, l'expression des récepteurs pour les IgG et la dégranulation. Ces deux derniers paramètres d'activité de la cellule sont également augmentés chez les éosinophiles humains. Par contre, aucun effet n'est observé sur les neutrophiles qu'ils soient de rat ou humains, ce qui souligne l'aspect sélectif des facteurs en cause vis-à-vis des éosinophiles. Cet effet stimulant, tout comme la spécificité pour cette classe de granulocytes n'est pas sans rappeler l'effet du médiateur mastocytaire qu' est le tetrapeptide ECF-A (Ala-Gly-Ser-Glu). Cette similarité est limitée au niveau fonctionnel puisque les deux molécules en cause semblent bien différentes. En effet alors que l'ECF-A

est une petite molécule, le facteur stimulant du SRP a un poids moléculaire supérieur, compte tenu de sa migration en filtration à travers un gel d'ACA 34 ou en ultracentrifugation. Par ailleurs, le facteur stimulant présent dans le SRP perd son activité après un chauffage à 100° pendant 3 minutes tandis que l'ECF-A est thermostable.

Cet effet stimulant est retrouvé dans les produits de sécrétion du parasite à tous les stades d'évolution chez l'hôte vertébré étudiés y compris les adultes mâles et femelles.

La thermosensibilité du facteur actif présent dans le SRP nous suggérait que l'effet stimulant pourrait être dû à des enzymes et plus particulièrement à des protéases dont on sait qu'elles sont présentes dans les produits de sécrétion des schistosomules. Cette hypothèse a été vérifiée par l'utilisation du Trasylol qui est un inhibiteur protéasique de large spectre d'activité. En effet, le pouvoir stimulant du SRP est très significativement diminué par le Trasylol (50 %) aussi bien dans les expériences de cytotoxicité vis-à-vis des schistosomules que dans celles de formation des rosettes.

La purification des protéases neutres des schistosomes par électrofocalisation préparative, gel filtration (ACA 34) ou les deux successivement met en évidence que l'activité stimulante des fonctions effectrices IgG dépendante des éosinophiles de rats vis-à-vis des schistosomules est toujours associée avec des protéases de spécificité collagénase puisque ces fractions peuvent hydrolyser des substrats tels que l'azocoll ou le peptide Z-Gly-Pro-Leu-Gly-Pro. Leur pouvoir hydrolytique est inhibé par le Trasylol, tout comme l'est le pouvoir stimulant sur les éosinophiles du SRP. Par ailleurs,

une collagénase extra-parasitaire comme la collagénase purifiée de Clostridium histolyticum reproduit l'effet stimulant sur la cytotoxicité anti-schistosomule IgG dépendante des éosinophiles. Ces résultats suggèrent donc que les facteurs thermolabiles présents dans le SRP responsables de l'activité sont des protéases neutres dont la spécificité collagénase est directement impliquée dans le processus activateur. Deux activités principales ont aussi été identifiées. L'une d'un poids moléculaire 45 à 68 000 ayant un pHi de 5,5 hydrolysant Z-Gly-Pro-Leu-Gly-Pro, l'autre plus neutre de poids moléculaire 10 à 25 000 hydrolysant l'azocoll et ayant un pHi de 6.2 à 6.8.

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Article N 6

Activation of Rat and Human Eosinophils by Soluble Factor(s) Released by *Schistosoma mansoni* Schistosomula

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The response of rat and human eosinophils and neutrophils to schistosome-released products (SRP) was examined. SRP activate rat eosinophils by enhancing both the IgG-dependent cytotoxicity for *Schistosoma mansoni* schistosomula and the expression of IgG-Fc receptors as measured by rosette assay. The increase by SRP of IgG-dependent rat eosinophil cytotoxicity was significantly correlated with the increase by SRP of eosinophil degranulation. SRP was also shown to induce both an increased expression of Fc γ receptors and the degranulation of human eosinophils. In contrast, no effect was obtained on human and rat neutrophil IgG Fc receptors or on neutrophil degranulation, suggesting a specificity of action on eosinophils. A similar effect on rat eosinophils was previously obtained with the mast cell mediator ECF.A (Ala–Gly–Ser–Glu). However, the parasite factor SRP lacked the chemotactic activity for eosinophils, was heat labile, and its molecular weight was higher than 2000. This indicates that the parasite-secreted factor was distinct from ECF.A tetrapeptides and has to be considered as a new mediator involved in the eosinophil activation. The role of this factor in schistosomiasis is discussed.

INTRODUCTION

Various experiments have clearly shown that normal eosinophils act as killer cells for *Schistosoma mansoni* schistosomula *in vitro* in the presence of specific antibodies from experimental or natural infections (1–4). This eosinophil-dependent cytotoxicity mechanism described in the rat model was shown to involve sequentially anaphylactic IgG2a (5) or IgE (6, 7) antibodies. The cytotoxic effect of rat eosinophils for antibody-sensitized schistosomula was dependent upon the presence of mast cells, whereas this cell type by itself was not able to kill parasites. The biological relevance of these observations accounts for the role of mast cell as a regulator cell of immunity in schistosomiasis through their interaction with eosinophils. In the absence of mast cells, the cytotoxic effect of eosinophils can be replaced by the addition of ECF.A[1] tetrapeptides, suggesting that this factor had an effect on eosinophils similar to unpurified, soluble mast cell mediators (8). It was further demonstrated that these mast cell products could act directly on the eosinophil membrane, as it was already suggested for complement receptors (9). Capron *et*

[1] Abbreviations used: MEM, Eagle's minimum medium; NRS, normal rat serum; IRS, immune rat serum; SRP, schistosome-released products; PEC, peritoneal exudate cells; ECF.A, eosinophil chemotactic factor of anaphylaxis; SRBC, sheep red blood cells E, erythrocyte; EA, erythrocyte antibody.

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al. (10) recently reported the enhancement by ECF.A tetrapeptides of the rat and human eosinophil Fc γ receptor as assessed by the EA rosette test. These observations could therefore be relevant to immunity in schistosomiasis, since the *in vivo* release of mast cell mediators could enhance both the expression of eosinophil Fc receptors and eosinophil cytotoxicity.

The present work was undertaken to explore the effect of parasite products on eosinophils and neutrophils. Results indicate that *S. mansoni* schistosomula themselves are able to stimulate both the cytotoxic function and the expression of Fc γ receptors of eosinophils whereas no effect was obtained on neutrophils.

MATERIALS AND METHODS

Media and Reagents

Eagle's minimum medium (MEM, DIFCO, Detroit, Mich.) and MEM supplemented with 1% heat-inactivated normal rat serum (MEM/NRS) were made to contain 100 IU penicillin/ml and 50 μg/ml streptomycin (7).

Parasite Life Cycle and Preparation of Schistosomula

A Puerto Rican strain of *Schistosoma mansoni* was used in this study (11). Schistosomula were obtained *in vitro* from cercariae by a mechanical procedure (12) in order to prepare products of incubation, or by the skin penetration method of Clegg and Smithers (13) for the cytotoxicity assays. Normal Fischer rats (Iffa Credo, l'Arbresle, France) infected with 1000 *S. mansoni* cercariae for 4 to 5 weeks were the source of immune rat serum (IRS). Normal rat serum (NRS) was obtained from uninfected Fischer rats.

Preparation of Schistosome-Released Products (SRP)

Mechanically prepared schistosomula were incubated for 4 hr at 37°C in MEM and washed six times to remove most of the cercarial enzymes. The schistosomula (10,000/ml) were then incubated for 16 hr at 37°C in MEM. The absence of bacterial contamination was controlled at the end of incubation, together with the viability of schistosomula, which was always superior to 95%. After centrifugation at 150*g* for 2 min, the schistosomula-free supernatant was recovered and considered as schistosome-released products (SRP). In some experiments, SRP was heated for 5 min at 100°C (heated SRP). To approximate the molecular weight of the active factor, SRP was filtered through UM2 membrane with a Amicon 8 MC microultracentrifugation unit.

Cell Populations

(1) Rat eosinophil-rich and mast cell-depleted populations. Rat eosinophils were prepared as previously described (7). Three days after the ip injection of 0.9% sterile physiologic saline to normal Fischer rats, they were killed using chloroform and the peritoneal cavities extensively washed with MEM/NRS. The PEC (2 to 4×10^7 cells from each rat) were pooled and centrifuged at 750*g* at 4°C and washed twice. Cells were resuspended at a concentration of 7×10^7 cells/ml in MEM/NRS, deposited onto plastic petri dishes, and incubated for 2 hr at 37°C

in 5% CO_2 atmosphere. The eosinophil-rich population consisted of the nonadherent PEC (35 to 80% eosinophils; 5 to 15% mast cells).

Peritoneal cells enriched in eosinophils and devoid of mast cells were prepared after centrifugation (400*g* for 15 min at room temperature) of the eosinophil-rich population upon metrizamide analytical grade (Nyegaard, Oslo, Norway) according to Lynch *et al.* (14). Briefly 1 ml (3 to 8×10^7 cells in MEM/NRS) was layered upon a 2-ml cushion of 22.5% (w/v) metrizamide containing 0.1% gelatin and 1% (w/v) deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.). After centrifugation the cell population called mast cell-depleted population (containing less than 1% mast cell/eosinophils) was recovered from the metrizamide-buffer interface and washed twice in MEM/NRS.

(2) Rat peritoneal neutrophils. Rat neutrophils (>90% purity) were obtained from the peritoneal cavity of Fischer rats 7 hr after the intraperitoneal injection of 10 ml of 10% proteose-peptone in saline as previously described by Capron *et al.* (10).

(3) Human eosinophils and neutrophils. Eosinophils and neutrophils were obtained from venous blood of human subjects and prepared according to the method previously described by Vadas *et al.* (15). This technique led to populations yielding more than 80% pure eosinophils or more than 90% pure neutrophils.

Cytotoxicity Assay.

The experimental procedure used was previously described (5). Fifty schistosomula were dispensed into each well of a flat-bottomed microtiter plate (Nunclon, Roskilde, Denmark) and incubated overnight at 37°C in a 5% CO_2 atmosphere with 100 μl of heat-inactivated IRS or NRS at a final dilution of 1:16 in MEM/NRS. Then schistosomula were washed with MEM/NRS and resuspended in 100 μl of the same medium. In each well was added 100 μl of MEM/NRS containing effector cells at a ratio of 6000 effector cells to one schistosomulum. SRP (10, 20 or 40 μl) was then added in each well of the cytotoxicity assay. In some experiments, the eosinophil chemotactic factor of anaphylaxis (ECF.A), Ala-Gly-Ser-Glu (Serva Laboratories, Darmstadt, West Germany), was added at a final concentration of 10^{-4} *M*, either unheated or heated at 100°C for 5 min. The percentage of cytotoxicity was measured after 48 hr contact by evaluating microscopically the number of living and dead schistosomula according to the previously described technique (5).

EA Rosetting Assay

Rat IgG-coated SRBC (EA) were prepared by mixing equal volumes of rat IgG anti-SRBC and a 2% solution of fresh SRBC as previously described (16). One hundred microliters of eosinophil or neutrophil populations (2×10^6/ml) was incubated for 60 min at 37°C under constant agitation with MEM, with ECF.A (10^{-4} *M* final concentration), or with 10, 20, or 40 μl SRP. After this incubation, the rosette assay itself was performed by adding 100 μl of nonsensitized SRBC (E) or EA (at a concentration of 3×10^8/ml). The tubes were then centrifuged at 100*g* for 10 min at 4°C and incubated for 30 min at 0°C. The pellet was gently resuspended and the cells were cytocentrifuged (Shandon-Elliot cytospin). The preparations were fixed in methanol and stained with Giemsa stain. All cells with

at least three adherent E were considered as rosettes. The percentage of 300 cells (eosinophils or neutrophils) that formed rosettes was numerated for each slide, and the percentage of rosettes obtained with control erythrocytes (E) was subtracted, giving the percentage of specific rosettes. Results were expressed as the mean of three replicates for each measurement.

Degranulation Test

One hundred microliters of rat or human eosinophils or neutrophils at a concentration of 2×10^6/ml were incubated with MEM, with ECF.A (10^{-4}final concentration), or with SRP (10, 20, or 40 μl) for 4 hr at 37°C in a 5% CO_2 atmosphere. In some experiments, heat-inactivated immune rat serum (4-week IRS) was added at a final dilution of 1/10. During this incubation period, the tubes were gently shaken three or four times in order to avoid cell adherence. The cell suspensions were then cytocentrifuged, and two cytocentrifuge preparations were prepared for each tube. These were fixed in methanol and treated with Giemsa stain. Cells were observed under a light microscope. The percentage of degranulation was established according to morphological criteria. In control preparations (eosinophils incubated with MEM), cells appeared intact with their stained intracellular granules. In some conditions of incubation, the eosinophil membrane seemed interrupted and numerous extracellular granules could be observed. Eosinophils were considered "degranulated" when they appeared larger than normal eosinophils, and when at least half of the total number of granules discharged into the exterior. All these results have been verified using the interference contrast microscopy (Polyvar, Reichert, West Germany) according to the technique recently described by Sher and Wadee (17). The percentage of these degranulated cells was evaluated by blind readings of 200 cells for each preparation. All values represented the mean of three to four experiments. In all conditions, the morphological aspect of neutrophils was unaltered.

Chemotaxis

The eosinophil migration was assessed using a method derived from Boyden's chamber technique (18).

RESULTS

Role of SRP on Rat Eosinophil Cytotoxicity

As previously described (8) the mast cell depletion of PEC significantly decreased eosinophil cytotoxicity against schistosomula opsonized with heat-inactivated IRS, whereas the addition of ECF.A (at a concentration of 10^{-4} *M*) restored the cytotoxic activity (Figs. 1A and B). The addition of soluble products released by schistosomula (SRP) to the mast cell-depleted rat eosinophil populations led to a significant increase of the percentage of cytotoxicity (Fig. 1B). The action of this parasite material was dose dependent and increased from 10 or 20 or 40 μl of culture medium, corresponding to the products released, respectively, by 100, 200, and 400 larvae for 16 hr. The enhancement was more significant when SRP was added to the mast cell-depleted population (Fig. 1B) than when it was added to the eosinophil-rich population (Fig. 1A). No cytotoxic effect was obtained when schisto-

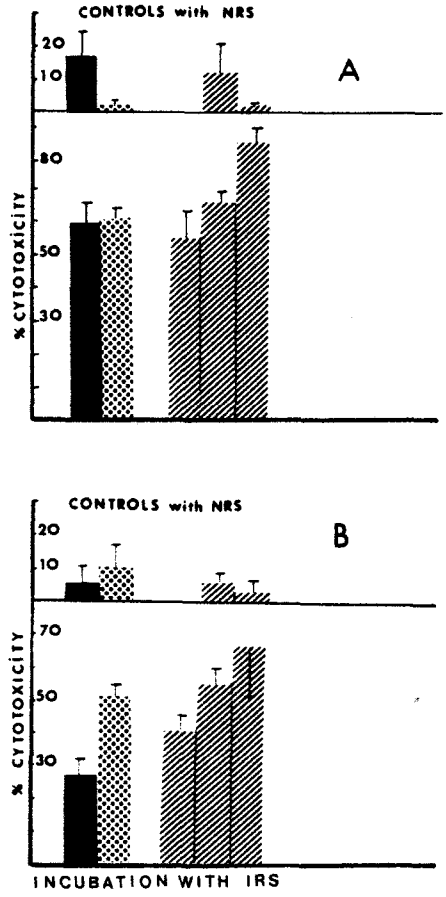

FIG. 1. Increase of rat IgG-dependent eosinophil cytotoxicity for schistosomula *in vitro*. (A) The effector cells consisted of eosinophil-rich populations (nonadherent PEC). (B) The effector cells consisted of a mast cell-depleted eosinophil population (containing less than 1% mast cells) after purification on metrizamide. All values represent the mean of three triplicate experiments. A significant increase was obtained with SRP (20 μl as reference): $P < 0.001$ (B). ■, MEM; □, ECF.A (10^{-4} *M*); ▨, 10, 20, and 40 μl of SRP added to effector cells, respectively.

somula were presensitized with NRS and further incubated in the presence of SRP, either with eosinophil-rich (Fig. 1A) or with mast cell-depleted populations (Fig. 1B). This indicates therefore, the requirement for antibody-mediated adherence of eosinophils to the schistosomula surface, and the absence of toxicity of SRP itself against the larvae.

Effect of SRP on Rat Eosinophil Degranulation

The eosinophil morphological degranulation was explored after 4 hr incubation at 37°C of rat eosinophil-rich population and MEM, 10^{-4} *M* ECF.A, SRP (10, 20, or 40 μl). Whereas eosinophils looked intact after incubation with MEM, a marked increase of degranulated cells was observed when eosinophils were incubated with ECF.A or with SRP, in a dose-dependent manner (Fig. 2A). Moreover, a significant correlation was obtained between the percentage of rat eosinophil

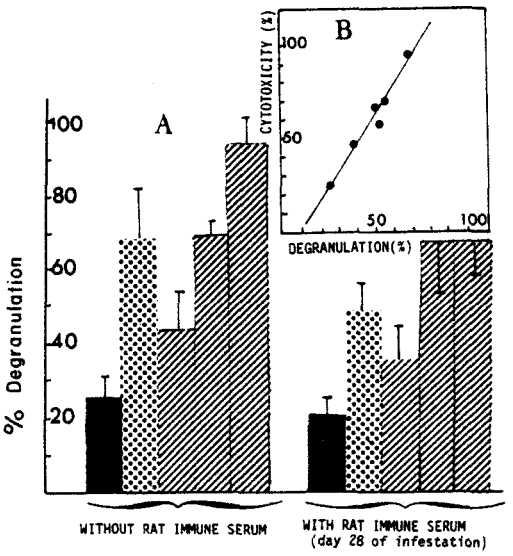

FIG. 2. Increase of rat eosinophil degranulation after 4 hr of incubation with SRP at several doses. (A) Incubation in the presence or the absence of rat immune serum. All values represent the mean of nine experiments. Significant increase with SRP (20 μl as reference): $P < 0.001$. (B) Significant correlation between the effect of SRP on rat IgG-dependent eosinophil cytotoxicity for schistosomula and on degranulation of eosinophils after incubation with SRP ($r = 0.96$; $P < 0.001$). ■, MEM; ▩, ECF.A ($10^{-4}M$); ▨, 10, 20, and 40 μl of SRP added to eosinophils, respectively.

degranulation and the IgG-dependent cytotoxicity of rat eosinophils for schistosomula, in the presence of the different doses of SRP which were tested ($r = 0.96$, $P < 0.001$, Fig. 2B). The same increase of degranulation by ECF.A or SRP was observed in the presence or in the absence of infected rat serum during the incubation period (Fig. 2A). This points to a direct action of SRP on eosinophil degranulation without the requirement of antibodies.

Enhancement of Rat Eosinophil-Fcγ Receptor by SRP

As previously shown for ECF.A tetrapeptides (10), the present study demonstrates the increasing capacity of rat eosinophils to bind to IgG-coated erythrocytes *in vitro* after incubation with SRP. Indeed, the incubation of rat peritoneal eosinophils with Ala–Gly–Ser–Glu (10^{-4} *M*) or SRP (10, 20, or 40 μl) markedly increased the expression of Fc receptors for rat IgG on the rat eosinophil membrane, as assessed by the EA rosette formation (Fig. 3). A dose-dependent increase of the percentage of eosinophils bearing IgG-Fc receptors was obtained for the two lowest doses tested. However, it should be noticed that with the highest concentration of SRP, the degranulation of eosinophils was important and therefore the decrease in the percentage of rosettes could be attributed to the cell degranulation.

Chemotaxis

As previously described by James and Sher for mouse PEC (19), no migration of rat PEC across the carbonate membrane filters in response to SRP was observed.

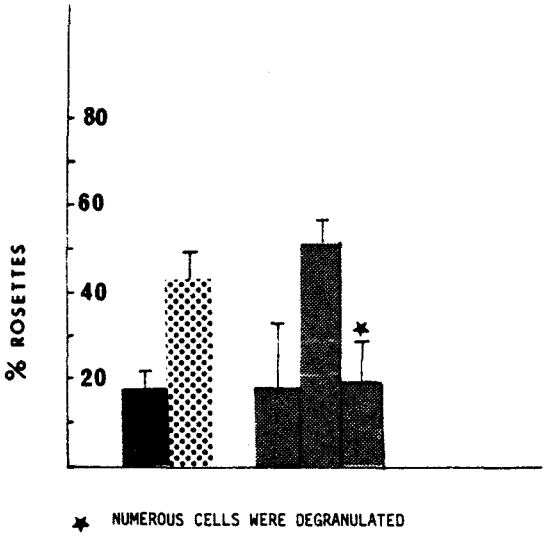

FIG. 3. Activation of the IgG-Fc receptor on rat eosinophils by SRP, assessed by EA rosette formation. All values represent the mean of four or five triplicate experiments. Significant increase with SRP (20 μl as reference): ($P < 0.001$). ■, MEM; ▨, ECF.A ($10^{-4}M$); ▩, 10, 20 and 40 μl of SRP added to eosinophils, respectively.

This fact suggests that schistosomula themselves did not secrete any component able to stimulate the eosinophil migration in these experimental conditions.

Heat Lability of the Parasite Factor Able to Activate Rat Eosinophils

To investigate the heat lability of the parasite factor, SRP previously heated for 5 min at 100°C was incubated with mast cell-depleted eosinophil populations and IRS-coated targets. As shown in Table 1, the level of cytotoxicity was significantly decreased and similar to the cytotoxicity for NRS-coated schistosomula. Similarly, heat-inactivated SRP becomes unable to enhance the expression of Fc γ receptor on eosinophil surface (Table 1). In the same conditions, the heat stability of ECF.A was controlled. The effect on IgG-dependent eosinophil cytotoxicity and on the eosinophil Fc γ receptor was similar when ECF.A was untreated or heated for 5 min at 100°C (Table 1). The heat lability of the parasite factor SRP seemed therefore to represent an essential difference with the heat-stable activity of ECF.A.

Estimation of the Molecular Weight of the Factor(s) Involved

In order to approximately evaluate the molecular weight (MW) of the parasite factor SRP two fractions (>2000 and <2000) obtained after filtration of SRP through UM2 Millipore dialysis membrane were compared to the unfractionated SRP (Table 2). While total SRP or more than 2000 SRP were able to increase both IgG-dependent cytotoxicity and EA rosettes, the fraction less than 2000 SRP failed to exhibit such an enhancing activity. This fact suggests that the parasite factor involved has a MW superior to 2000. In addition, preliminary results on the purification of SRP show that its MW is between 25,000 and 65,000 as determined by gel filtration on Ultrogel ACA-34. Considering the low MW of the ECF.A

TABLE 1

Heat Lability of the Effect of Schistosomula-Released Products (SRP) on IgG-Dependent Eosinophil Cytotoxicity for Schistosomula and on Eosinophil EA Rosette Formation[a]

Rat eosinophils incubated with	Cytotoxicity (%)		Specific rosettes (%)[b]
	IRS	NRS	
Medium (MEM)	14.8 ± 7.3	15.2 ± 10.4	13.33 ± 5.3
SRP	50.7 ± 6.2	13.3 ± 12.7	45.1 ± 6.5
Heated SRP[c]	20.1 ± 7.8[d]	14.0 ± 10.8	7.8 ± 4.0[e]
ECF.A	43.6 ± 3.6	16.7 ± 4.0	41.6 ± 9.3
Heated ECF.A[c]	48.1 ± 4.0	9.9 ± 7.8	39.2 ± 5.8

[a] All values represent the mean of five triplicate experiments (±SE).
[b] The percentage of specific rosettes was obtained by subtracting the percentage of rosettes obtained with control erythrocytes (E). See Materials and Methods.
[c] 100°C for 5 min.
[d] Significantly less important when compared to nonheated SRP ($P < 0.01$).
[e] Significantly less important when compared to nonheated SRP ($P < 0.001$).

tetrapeptides (300 to 500), it seems therefore unlikely that the parasite factor involved in eosinophil activation is an ECF.A molecule synthesized by the larvae.

Role of SRP on Human Eosinophils: Comparison with Human and Rat Neutrophils

In order to study the role of SRP on human eosinophils, a similar protocol exploring Fc γ receptors and eosinophil degranulation was used. As observed in the case of rat eosinophils, SRP significantly increased both the expression of Fc γ receptors as measured by the EA rosette formation, and the degranulation after a 4-hr incubation (Table 3). A similar effect on EA rosettes was obtained with

TABLE 2

Molecular Weight Estimation of the Active Factor Present in Schistosomula-Released Products (SRP) Tested in IgG-Dependent Eosinophil Cytotoxicity for Schistosomula and Eosinophil EA Rosette Formation[a]

Rat eosinophils incubated with	Cytotoxicity (%)		Specific rosettes (%)[b]
	IRS	NRS	
Medium (MEM)	17.2 ± 5.1	8.3 ± 3.2	5.0 ± 2.4
SRP	49.1 ± 5.8	14.2 ± 12.7	33.7 ± 3.8
SRP > 2000	52.5 ± 6.3[c]	ND	20.8 ± 8.2[c]
SRP < 2000	25.6 ± 6.2[d]	ND	3.6 ± 2.1[e]

[a] All values represent the mean of three to five triplicate experiments (±SE); ND, not done.
[b] See Materials and Methods.
[c] No significant difference when compared to total SRP.
[d] Significantly decreased when compared to total SRP ($P < 0.02$).
[e] Significantly decreased when compared to total SRP ($P < 0.05$).

TABLE 3

Effect of SRP on EA Rosette Formation and Degranulation of Human Eosinophils: Comparison with Human and Rat Neutrophils

Cells incubated with	EA rosettes (%)[a]			Degranulation (%)[b]	
	Hu Eos	Hu Neutros	Rat Neutros	Hu Eos	Hu Neutros
Medium	32.6 ± 6.6	14.0 ± 2.0	19.3 ± 6.7	13.3 ± 4.6	0.7 ± 0.6
ECF.A	44.6 ± 9.8	22.6 ± 2.4	22.6 ± 5.7	28.7 ± 3.5	2.0 ± 1.1
SRP	48.6 ± 4.0[c]	7.3 ± 2.4[d]	9.3 ± 0.6	80.7 ± 1.7[e]	1.3 ± 0.6

[a] EA rosette formation was estimated by using E sensitized with rat antibody. The various cell preparations were incubated for 1 hr at 37°C with medium (MEM), ECF.A (10^{-4} *M* final concentration), or SRP (20 μl). Results represent the mean of triplicate experiments ± SE.

[b] The percentage of degranulation was measured microscopically after 4 hr incubation at 37°C of the cell preparations with the different products (mean ± SE of triplicate experiments).

[c] Significantly higher than after incubation with medium ($P < 0.05$).

[d] Significantly lower than after incubation with medium ($P < 0.05$).

[e] Significantly increased when compared to medium ($P < 0.001$).

ECF.A, as previously reported (10), but the effect of ECF.A on human eosinophil degranulation was more limited.

In contrast, no enhancing effect of SRP on neutrophil Fc receptors or on neutrophil degranulation could be detected, as it was already observed in the case of ECF.A and neutrophil Fc receptor (10). Moreover a significant decrease in the percentage of EA rosettes was noticed when human neutrophils were incubated with SRP (Table 3).

DISCUSSION

The present results show that excretion–secretion products of *S. mansoni* schistosomula cultivated *in vitro* during a 16-hr period can increase the antibody-dependent schistosomulicidal activity of rat eosinophils. Constituants of some helminth parasites such as soluble extracts of *Taenia taeniaeformis* cysticerci (20) or perienteric fluids of Ascaris (21, 22) have been shown to stimulate both eosinophil and neutrophil migration. More recently James and Sher (19) have reported that schistosomula activate mouse leukocyte migration by means of a lymphokine produced by prestimulated lymphocytes. On the other hand, complement activation by the parasite (23–26) could be likewise one means of recruiting leukocytes toward the target.

The present work confirms that excretion–secretion products of schistosomula are unable to stimulate eosinophil migration as already demonstrated for mouse eosinophils (19). In contrast, these products were shown to increase in parallel the capacity of degranulation and the IgG-dependent cytotoxic effect of rat eosinophils and also the degranulation of human eosinophils. These results have to be related to the elegant studies by Butterworth *et al.* (27), suggesting that the stable adherence of eosinophils to IgG-coated schistosomula and subsequent damage of the larvae by these cells are attributable to the granule contents of eosinophils. But in this work, no evidence allowed the identification of the factor(s) responsible for

eosinophil degranulation. Our experiments suggest that after antibody-mediated adherence of eosinophils to schistosomula, products secreted onto or present on the parasite surface lead to eosinophil degranulation. The degranulation of the cell could occur at least partly in relation to the binding (IgG mediated). But since an increase of eosinophil degranulation was also observed in the absence of antibody, this suggests that SRP could likewise act directly on eosinophils without interaction with antibodies. Then the release of eosinophil granule contents such as the major basic protein (MBP) could damage the larvae tissue (27). In contrast, recent studies have shown that neutrophils, although able to adhere to opsonized schistosomula, did not degranulate or induce damage (28). The present results allow therefore the clarification of this point by showing that, whereas parasite components could induce human and rat eosinophil degranulation, no effect was detected on neutrophils.

The present study displays moreover an increased expression of IgG-Fc receptor on human and rat eosinophils in the presence of SRP as already observed with ECF.A tetrapeptides (10). It has been previously reported that the peptides resulting from the cleavage of bound IgG upon schistosomula by their Fc fragments are able to modulate the macrophage activity (29, 30). In this case, the parasitic material acted indirectly on cytotoxic macrophages by means of secreted proteases (31) and the regulatory process involved host antigen such as nonspecific IgG. It is shown here that parasite-secreted material is directly able to increase the eosinophil activity. The present results clearly show that SRP exhibits an eosinophil-enhancing capacity similar to the one previously described for ECF.A tetrapeptides (10). The possibility that schistosomula could secrete ECF.A tetrapeptides is therefore to be considered. Two observations are however against this hypothesis. The molecular weight of the molecule involved in SRP is higher than 2000, whereas the MW of ECF.A is approximately 500. Moreover the parasitic factor seems heat-labile whereas ECF.A is heat-stable when tested in IgG-dependent rat eosinophil cytotoxicity and in EA rosette formation. Tai and Spry recently demonstrated that eosinophils incubated with Pronase or trypsin developed an increased capacity to form EA rosettes (32). This indicates that the expression of the eosinophil IgG receptor can be modulated not only by ECF.A, but likewise by biologically active molecules with higher MW. The presence of ECF.A in SRP cannot be definitely ruled out but the enhancing effect of schistosomula products on eosinophil activity is likely to be due to another process.

In conclusion, we have demonstrated that schistosomula are able to increase the eosinophil-dependent schistosomulicidal activity *in vitro* by the action of a secreted factor, the chemical properties of which are presently under investigation. The effects of this parasite factor are not restricted to rat eosinophils, since human eosinophils were also activated in the same conditions. The inefficiency of SRP on neutrophils could explain at least partly why eosinophils but not neutrophils are cytotoxic for schistosomula (28). Therefore, it seems possible that the parasite by means of immunoregulatory substances might interfere with the host immune response and could thus control the host–parasite relationship.

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Article N 7

Running title : Enhancement of eosinophil activity by S. mansoni proteases

Full title : Enhancement of eosinophil effector function by soluble factors released by S. mansoni : Role of proteases.

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[4]Abbreviations used in this paper : SRP, schistosome released products ; NRS, normal rat serum ; IRS, immune rat serum ; E, erythrocyte ; EA, erythrocyte antibody ; MEM, Eagle's minimum medium ; ECF-A, eosinophil chemotactic factor of anaphylaxis ; BAW, butanol acetic acid, water (4 : 1 : 5 by vol.)

ABSTRACT

Schistosomulum released products (SRP) have previously been shown to enhance both expression of rat and human eosinophil Fc receptor and IgG-dependent cytotoxicity. The present work provides additional evidence for the secretion of eosinophil enhancing factors by schistosomula and other developmental stages of schistosomes, including adult worms. The heat lability as well as the strong inhibition of the stimulating activity of SRP by the protease inhibitor Trasylol suggest that thermolabile proteases secreted by the parasite are involved in this mechanism. The purification of the schistosome proteases by preparative isoelectric focusing and gel filtration showed that neutral proteases able to hydrolyse the collagenase substrates Azocoll and Z-gly-pro-leu-gly-pro are able to significantly enhance eosinophil effector functions. Purified Clostridium histolyticum collagenase was also able to mimic the enhancing effect of schistosome proteases, suggesting involvement of a collagenase like activity of the enzymes in the eosinophil stimulation.

INTRODUCTION

Evidence has been brought both in the human and in experimental models, of different mechanisms involving eosinophils as effector cells of immunity, in various systems of cell-antibody cooperation, leading to the killing of Schistosoma mansoni larvae (1 - 4). The characterization of the immunoglobulin classes involved in rat eosinophil cytotoxicity revealed the dependence on anaphylactic antibodies (5 - 7). Moreover, the study of this eosinophil-mediated mechanism in the rat, led to the observation that besides eosinophils, peritoneal mast cells, though unable by themselves to kill schistosomula, participated as accessory cells in the induction of eosinophil mediated cytotoxicity (8). In the absence of mast cells, the cytotoxic effect of rat eosinophils could be restored by products of mast cell degranulation. Among these products, the role of the eosinophil chemotactic factor of anaphylaxis (ECF.A) was shown to be significant (9).

Recently, we demonstrated that soluble factor(s), named SRP (Schistosome Released Products) from Schistosoma mansoni schistosomula enhance rat and human eosinophil activity, particularly their antibody-dependent cytotoxicity against schistosomula and their expression of the Fcγ receptors (10). Thus, SRP exhibited an enhancing capacity on eosinophils, similar to the one described for ECF.A tetrapeptides. The differences in heat-sensitivity and in molecular weight between SRP and ECF.A however indicated that the activity of SRP was not due to ECF.A like material liberated by the parasite but was likely due to another factor. SRP was without effect on neutrophils and the specificity for eosinophils of parasitic secretory substances can be considered as one means developed by the parasite to interfere with the host immune response.

We have previously shown that schistosomula secrete proteinases able to cleave IgG bound to the surface of the larvae and the peptides resulting from this cleavage modulate macrophage activity (11). The heat lability of SRP and the presence of the proteases in the secretory products of schistosomula led us to study these enzymes. In the present paper, we show that the enhancing activity on rat eosinophils in SRP is attributable to secreted proteases with collagenase-like activity and is found at different stages of evolution of the parasite in its mammal host.

MATERIALS AND METHODS

- Media and reagents. Eagle's minimum medium (MEM, Difco, Detroit, Mich.) and MEM supplemented with 1 % heat-inactivated normal rat serum (MEM/NRS) also contained 100 IU penicillin/ml and 50 µg/ml streptomycin (7). The protease inhibitor Trasylol was purchased from Sigma chemical Co, St Louis, Mo, USA. ACA-34 Ultrogel was provided by Pharmindustrie, France. Ampholines were purchased from LKB, Bromma, Sweden.

Parasite life cycle and preparation of schistosomula. A Puerto-Rican strain of Schistosoma mansoni was used throughout the study (12). Schistosomula were obtained in vitro from cercariae by a mechanical procedure (13) in order to prepare products of incubation or by the skin penetration method of Clegg and Smithers (14) for the cytotoxicity assay.

Inbred Fischer rats (Iffa Credo, L'Arbresle, France) exposed percutaneously to 1000 S. mansoni cercariae 4 to 5 weeks previously were the source of immune rat serum (IRS). Uninfected rats of the same origin were the source of normal serum (NRS) and were used for the collection of peritoneal cells.

- Recovery of lung stage larvae, 20-day-old and adult worms. Lung stage schistosomula were obtained from hamsters infected 6 days previously with 4000 cercariae. In order to separate the parasites from the contaminating tissue, the lungs were finely minced and then filtered through gauze.

The 20-day-old parasites and the adult worms were collected by perfusion of the liver and the mesenteric veins 20 or 40 days respectively after the infestation of hamsters (1000 cercariae per animal). The living parasites were then washed at least 6 times in Hank's balanced salt solution for three hours.

Preparation of schistosome-released products (SRP). Mechanically prepared or lung stage schistosomula, 20-day-old and adult worms were incubated for 4 hr at 37°C in MEM and then extensively washed in the same medium. The parasites, 10,000/ml for mechanically prepared and lung stage schistosomula, 100/ml for 20-day-old and 20/ml (10 males + 10 females) for adult worms were then incubated for 16 hr at 37°C. The absence of bacterial contamination was controlled at the end of incubation, together with the viability of the parasites which was always superior to 95 % for the larvae and was 100 % for the adult worms. The parasite-free supernatant was recovered after centrifugation at 150 x g for 2 min and refered to as schistosome released products (SRP).

Preparation of adult worm extracts. The adult worms were first treated by sonication (microsonde 3 times, 5 sec 70 watts, sonifier B-12 Branson Sonic) at 0°C in 0.1 M phosphate buffer pH 7.2, in order to destabilize the membrane. This treatment was followed by homogeneization using a hand rotated Potter-Elvehjem homogenizer with a tight fitting teflon pestle. The temperature was maintained at 4°C during this treatment. The homogenates were then centrifuged at 20,000 x g for 1 hr at 4°C. The lipids were removed from the surface and the supernatant was considered as the crude extract of adult worms.

Cell populations. Rat eosinophils were prepared as previously described (7). Three days after the I.P. injection of 0.9 % sterile physiologic saline, the peritoneal cavities of normal Fischer rats were washed with MEM/NRS. The peritoneal exudate cells were pooled and centrifuged at 750 x g at 4°C and washed twice. After resuspension at a concentration of 7 x 10^6 cells/ml in MEM/NRS, PEC were seeded in plastic petri dishes, incubated for 2 hr at 37°C in a 5 % CO_2 atmosphere and the non-adherent cells were recovered.

Peritoneal cells enriched in eosinophils and devoid of mast cells were prepared according to the method described by Lynch et al. (15). Briefly, 1 ml of non-adherent cells (3 to 8 x 10^7 cells in MEM/NRS) was layered onto a 2 ml cushion of 22.5 % (w/v) metrizamide containing 0.1 % gelatine and 1 % (w/v) deoxyribonuclease (Worthington Biochemical Corporation, Freehold, NJ). After centrifugation, the cell population recovered from the interface was washed twice in MEM/NRS. It constituted the eosinophil-rich population (90 to 95 % of eosinophils) and was depleted in mast cells (less than 1 %).

EA Rosetting Assay. Rat IgG-coated SRBC (EA) were prepared by mixing (w/v) a 2 % solution of fresh SRBC and rat IgG anti-SRBC (16). One hundred microliters of mast cell-depleted eosinophil population (4 x 10^6 cells/ml) were incubated for 1 hr at 37°C under constant agitation with MEM or with SRP (10 to 20 µl). The rosette assay was then performed by adding 100 µl of EA or non sensitized SRBC (E) at a concentration of 3 x 10^8 cells/ml. After centrifugation at 100 x g for 10 min at 4°C, the tubes were incubated for 30 min at 0°C. The cells were resuspended and cytocentrifuged. After fixation in methanol the preparation was stained with Giemsa stain. At least, 200 eosinophils were counted. The cells that formed rosettes (at least three adherent erythrocytes by eosinophil) was determined for each slide. The percentage of rosettes obtained with E as control was subtracted, giving the percentage of specific EA rosettes. Results were expressed as the mean of three experiments.

Degranulation test. The experimental procedure used was previously described (10). Briefly, 100 µl of rat eosinophils at a concentration of 2 x 10^6 cells/ml were incubated with MEM or SRP (20 µl) for 4 hr at 37°C in a 5 % CO_2 atmosphere. During the incubation period, the tubes were gently shaken

three or four times in order to prevent cell adherence. The cell suspensions were then cytocentrifuged. Each preparation was fixed in methanol and treated with Giemsa stain. The percentage of degranulation was established according to morphological criteria under a light microscope. Eosinophils were considered as degranulated when they appeared larger than normal, their membrane was interrupted and at least half of their intracellular granules were discharged (10). The percentage of degranulated cells was evaluated by blind readings of 200 cells at least for each preparation. All values represent the mean of three experiments or more.

Cytotoxicity assay. As previously described (5), 50 skin-prepared schistosomula were dispensed into each well of a flat-bottomed microtiter plate (Nunclon, Roskilde, Denmark) and incubated at 37°C in a 5 % CO_2 atmosphere with 50 µl of heat-inactivated IRS or NRS at a final dilution of 1 : 16 in MEM-NRS. After overnight incubation, 100 µl of the mast cell-depleted eosinophil population (6000 effector cells to 1 target) and 20 µl SRP were simultaneously added to each well of the cytotoxicity assay. The percentage of cytotoxicity was measured after 48 hr contact between effector cells and targets by microscopical examination. Schistosomula were counted as dead if they were immobile, granular and opaque and surrounded by 1 or several layers of cells.

Protease inhibitors. In some experiments, SRP was treated with Trasylol (aprotinin) containing 10260 KIU/mg (Sigma Chemical Co, St Louis, Mo). Three hours before its utilization, 1200 KIU of protease inhibitor were dissolved in 1 ml of SRP. Trypsin inhibitory capacity indicated that 0.80 mole trypsin was inhibited per mole of Trasylol.

Enzyme assays. Azocoll (Sigma Chemical Co, St Louis, Mo) was used at a 2 % concentration in 0.1 M Sodium phosphate buffer, pH 7.2, containing 0.01 % sodium azide. Two ml of substrate and 50 µl of parasite extract were incubated at 37°C for 6 - 10 hr under agitation. The hydrolysis of Azocoll was measured directly at 540 nm. Z-gly-pro-leu-gly-pro (Sigma Chemical Co, St Louis, Mo) was solubilized at 1 mg/ml in the same buffer. The hydrolysis of 50 µl was carried out by incubating the substrate with 10 to 20 µl parasite extract at 37°C for 6 hr. In each case a blank of hydrolysis was made by adding immediately a drop of concentrated acetic acid (30 % V/V). At the end of the incubation period, hydrolysis was stopped in the same manner.

The hydrolysates of Z-gly-pro-leu-gly-pro, with parasite extracts was spotted onto Whatman paper n°3 (46 x 57 cm). Controls with parasite extract and substrate alone were included. Elution was carried out for 10 hr at room temperature in butanol : acetic acid : water, 4 : 1 : 5 (BAW). The dried spots were revealed using a 1 % ninhydrin solution in acetone.

Gel filtration chromatography. In order to separate proteins (particularly the proteases) according to their molecular weights, crude adult extracts (10 to 20 mg total proteins) were filtered through a column (2 x 90 cm) containing ACA-34 Ultrogel (Pharmindustrie, France). The absorbance of the eluate was monitored at 280 nm by a LKB Uvicord S (Bromma, Sweden).

Isoelectric focusing. Preparative horizontal bed isoelectric focusing was carried out using an LKB multiphor apparatus (Bromma, Sweden). The gel bed was prepared with 100 ml of Sephadex G75 containing 2.5 % carrier ampholines, pH range 3.5 - 10 and the crude adult worm extract (10 to 20 mg total proteins). Focusing was performed for 16 hr at 8 watts constant

power. The gel bed was then divided into 30 sections using an LKB template. Each gel fraction was then placed in a 10 ml syringue barrel fitted with a porous plastic sinter and eluted with 3 ml water. After pH determination, the fractions were dialyzed overnight against 0.1 M Sodium phosphate buffer pH 7.0 containing 0.15 M NaCl to remove ampholines.

Statistical study. In the rosette assays, the net change was calculated as the difference between the eosinophil specific rosettes after incubation with MEM and with SRP. In the cytotoxicity assay, the net change was calculated by subtracting the values obtained with NRS and IRS in the same experimental conditions. A one-tailed Student's t test was used.

RESULTS

Enhancement of eosinophil cytotoxic activity by SRP from different development stages. The effect of soluble products released by schistosomula (day 0), lung stage schistosomula (6-day-old), liver stage schistosomes (20-day-old) and adult worms (40-day-old) on mast cell-depleted rat eosinophils has been studied. In all cases, the addition of SRP (20 µl) led to a significant increase in the eosinophil cytotoxicity against schistosomula opsonized with heat-inactivated IRS (Table 1). In contrast, no cytotoxic effect was observed when the targets were presensitized with NRS and further incubated in the presence of SRP. This indicates the absence of toxicity of SRP itself against the larvae, whatever the source of SRP is. As previously demonstrated, eosinophil cytotoxicity required both antibody-mediated adherence and degranulation of the cells (4). For this reason, the effect of SRP on the expression of eosinophil-Fcγ receptors and cell degranulation was also studied (Table 1). The results revealed that SRP produced by each developmental stage increased both the expression of Fc γ receptors and the degranulation of rat eosinophils.

Heat lability of parasite derived factors able to activate eosinophil EA rosette formation and IgG dependent cytotoxicity. We have previously shown that SRP produced by schistosomula loses its eosinophil enhancing activity when heated for 5 min at 100°C. Results shown in Table 2 and Table 3 indicate that SRP from adult worms (males + females) heated in the same manner became unable to increase the expression of IgG-Fcγ receptor of rat eosinophils as well as the IgG dependent anti-schistosomula cytotoxicity. Moreover, SRP obtained from either male or female adult worms were equally able to enhance the EA rosette formation. In each case, this activity was

destroyed by heating. Heat lability seems thus to represent a common feature of the enhancing parasite factor(s) in SRP whichever the developmental stage.

Inhibition of the enhancing activity of SRP by Trasylol. Trasylol (aprotinir also referred to as kallikrein inactivator, is known to bind to and to inactivate a variety of proteases such as trypsin chymotrypsin, cathepsin and papain. In order to study the effect of Trasylol-treated SRP on the eosinophil activity, the protease inhibitor was added to SRP (1200 KIU per ml of SRP) three hours before the experiments. Tables 2 and 3 indicate that the prior incubation of SRP with Trasylol strongly inhibited its enhancing effect on eosinophil-Fc γ receptor expression and on IgG-dependent anti-schistosomula eosinophil cytotoxicity. No cytotoxic effect was observed when the targets were presensitized with NRS and further incubated in the presence of Trasylol-treated SRP and the eosinophil-rich population. Moreover, Trasylol itself did not interfere with the cytotoxic process, since in the absence of SRP, Trasylol alone used at the same concentration had no significant enhancing or inhibiting effect. These results indicate therefore that thermolabile proteases present in SRP could be involved in the enhancement of eosinophil activity.

Enhancement of the expression of rat eosinophil-Fc γ receptor by proteases of adult worms, separated by preparative isoelectric focusing (pH range 3.5 - 10). The neutral proteinase activities of a crude enzymatic extract (10 mg total proteins) from adult worms, which hydrolyse Azocoll and Z-gly-pro-leu-gly-pro as substrates, were tested after separation by isoelectric focusing (pH range 3.5 - 10). These enzymes have previously been characterized (17) and they represent two of the major neutral protease activities of Schistosoma mansoni. Z-gly-pro-leu-gly-pro and Azocoll are considered

as collagenase substrates. The former was hydrolyzed by an enzyme activity with an isoelectric point of 5.4 to 5.6 (Fig. 1A). Azocollytic activity was resolved into two overlapping peaks (pI 6.5 and 6.7) (Fig. 1B).

The separated proteases were tested for their capacity to enhance rat eosinophil Fcγ receptors. The proteases able to hydrolyse Z-gly-pro-leu-gly-pro (evidenced by liberation of leucine) and the acidic azocollytic activity both enhanced eosinophil EA rosette formation (25 to 38 %) (Fig. 1C). A basic proteinase exhibiting an aminopeptidase activity previously characterized by its particular ability to cleave the dipeptide leu-gly (17) also increased the percentage of rosettes although to a lesser extent. In contrast, the aminopeptidase able to hydrolyse leucine-p-nitroanilide (PI 5.8, 5.9) or the caseolytic enzyme (PI 6.1) failed to exhibit such an enhancing effect (17).

Enhancement of rat eosinophil-Fcγ receptor and of anti-schistosomula cytotoxicity by proteases of adult worms separated by gel filtration. In an attempt to separate the neutral proteases from adult worms according to their molecular weight, a concentrated crude adult worm extract (10 mg protein) was subjected to Ultrogel ACA-34 chromatography. Six column fractions were assayed for their proteolytic activity against Z-gly-pro-leu-gly-pro and for their azocollytic activity (Fig. 2A). Two peaks exhibited azocollytic activity. The first corresponded to high molecular weight proteases (F2, m.w. superior to 230,000), the other to proteases of a molecular weight of about 20 to 68 000 (F4). Enzyme activity able to cleave Z-gly-pro-leu-gly-pro was also located in the fourth fraction along with that hydrolysing Azocoll substrate (F4). These fractions were tested for their capacity to enhance both eosinophil Fcγ receptor expression and IgG

dependent anti-schistosomula eosinophil cytotoxicity (Fig. 2B and 2C). The proteinase activities which hydrolyse Z-gly-pro-leu-gly-pro and Azocoll both enhanced eosinophil activity. Indeed, the addition of the two separated fractions that contained proteases led to a 20 % increase in EA rosette formation with F2 and 55 % with F4. In parallel, the same fractions led to an increase in the eosinophil-dependent anti-schistosomula cytotoxicity (25 % and 35 % respectively).

These results indicate that at least part of the eosinophil enhancing factor(s) of the adult worms are copurified either after isoelectric focusing or after molecular sieving with the proteases able to hydrolyse the two collagenase substrates, azocoll and Z-gly-pro-leu-gly-pro.

Biochemical characterization of the proteases involved in the enhancement of eosinophil activity. In order to characterize more precisely the molecules involved in eosinophil activation, they were purified from adult worm homogenates, first by isoelectric focusing in the pH range 3.5-10 (20 mg protein Fig. 3), followed by ACA 34 Ultrogel chromatography and tested using the same substrates as above (Fig. 4). Two fractions F.A and F.B were separated after isoelectric focusing. Three fractions A1, A2 and A3 were able to hydrolyze Z-gly-pro-leu-gly-pro after chromatography of F.A, A2 exhibiting the major activity. Three azocollytic fractions : B1, the first and major peak, B2 and B3 were obtained after chromatography of F.B. The six fractions purified according to their pI, their molecular weight and their proteolytic activities were then tested at a final protein concentration of 2 µg/ml in the anti-schistosomula eosinophil cytotoxicity assay (Table 4). Two of these fractions enhanced eosinophil cytotoxicity : A2 (m.w. 45 to 68 000, pI 5.5, able to hydrolyse Z-gly-pro-leu-gly-pro) and B3 (m.w. 10 to 25 000, pI 6.2 to 6.8, cleaving azocoll). These results reproduced those obtained in the

previous experiments using crude extracts or fractions separated only by a simple isoelectrofocusing or molecular sieving. A1 and B1 (m.w. superior to 230,000) also exhibited an enhancing activity but to a lesser extent. These results confirmed that using two different physicochemical methods of separation, the eosinophil enhancing activity remains associated with the enzymes able to cleave the collagenase substrates.

Inhibition by Trasylol of purified schistosome proteases able to enhance eosinophil activity. As shown above, SRP pretreated with Trasylol was unable to enhance the expression of eosinophil Fcγ receptor and the cytotoxic effect of this cell against schistosomula. Since purified adult worm proteases exhibited the same enhancing effect as crude SRP, the inhibitory role of Trasylol was assessed on the hydrolysis of Azocoll and Z-gly-pro-leu-gly-pro by these proteinases. The azocollytic acticity of an enzymatic crude extract was only partially inhibited by Trasylol (40 %). In contrast, the azocollytic activity of fraction B3 was strongly decreased (80 to 90 %) in the presence of Trasylol (2000 KIU/ml for an enzymatic extract of 5 mg protein/ml). The liberation of leucine by the fraction A2 after hydrolysis of Z-gly-pro-leu-gly-pro was also inhibited in the presence of the enzyme inhibitor. In this case, Trasylol only partially inhibited the proteolysis, since a low level of liberated leucine was still detected after 10 hr of hydrolysis but to a lesser extent that without the inhibitor. These results emphasize that the active molecules present in SRP and the purified proteolytic fractions share the same biological activities and were similarly inhibited by the protease inhibitor Trasylol. To support these results collagenase activities inhibited by Trasylol have been found in excretory-secretory products of schistosomula and adult worms (Cesari et al., manuscript in preparation).

Effect of Clostridium histolyticum collagenase on IgG-dependent eosinophil cytotoxicity. In order to define whether the collagenase specificity of the parasite proteases described above was an important feature of the eosinophil enhancing activity of SRP, a purified bacterial collagenase (type IV-S Sigma Chemical Co, St Louis, Mo) was added to the cytotoxicity assay (Table 5). The addition of the collagenase significantly enhanced IgG-dependent eosinophil cytotoxicity at the lowest concentration used (100 μg/ml). In contrast, eosinophils were not stimulated when higher enzyme concentration were used (500 μg/ml). These results suggest that bacterial collagenase as well as schistosome proteases able to cleave collagenase substrates, both enhance eosinophil effector function.

DISCUSSION

In a previous report, we showed that excretory-secretory products released by S. mansoni schistosomula cultivated in vitro during 16 hr were able to increase eosinophil activity, particularly in the capacity of this cell to kill young schistosome larvae (10). The present study was undertaken to define the time course of the secretion of this enhancing material during the development of the parasite in its mammalian host, and to determine its chemical nature. The results presented here demonstrate that not only are the products secreted by schistosomula able to stimulate some essential eosinophil functions such as the Fcγ receptor expression or schistosomulicidal activity, but the enhancing factor is found in the secretion products of later developmental stages. Indeed, the soluble products released by 6- and 20- day old larvae as well as the adult worms also enhance eosinophil activity assessed by these criteriae. This indicates that adult parasites in the infected host are potentially able to enhance eosinophils in their capacity to kill young larvae, which penetrate into the infested host, in cooperation with specific antibodies. The need for antibodies for the eosinophil effector function and the fact that only the young schistosomula are killed in vitro by these cells (4) suggests that parasite-secreted factors are able to prevent multiple infections by stimulating one of the main effector cells involved in anti-schistosome immunity. The enhancement in the expression of eosinophil Fcγ receptor could lead to an increase in antibody-dependent adherence to the larvae and thus allows the killing of the schistosomula after degranulation of the effector cells. In addition, the eosinophil enhancing activity released by adult schistosomes is abolished by heating (100°C for 5 min). While the heat-lability of products released

by schistosomula has already been described (10), the present results show that the enhancing activity of products released by adult worms is also destroyed by heating, suggesting a biological analogy between the products secreted by the parasite at these two developmental stages.

The heat lability of the biologically active material present in SRP suggests that the involved molecule could act through an enzymatic activity. It was previously reported that schistosomula secrete proteases able to modulate macrophage activity by means of peptides liberated after hydrolysis of IgG (19). The involvement of such proteases in the eosinophil stimulatory activity of SRP was thus considered. Trasylol (aprotinin), an inhibitor known to inactivate a variety of proteases was used as a probe for studying the protease involvement in the eosinophil enhancing activity of SRP. At a concentration of 1200 kallikrein inactivating units per ml, this inhibitor significantly decreases the effect of SRP. This inhibition reduces 50 to 80 % of the enhancing effect either on the formation of specific EA rosettes or on anti-larval cytotoxicity. This suggests that proteases present in SRP play a major role in the enhancing effect on eosinophils. To comfort this hypothesis, the activating capacity of schistosome neutral proteases was tested after their partial purification by preparative isoelectric focusing and/or gel filtration. The results show that purified schistosome proteases are effectively able to enhance eosinophil activity and more particularly those hydrolysing collagenase substrates. These proteases are also inhibited by Trasylol. Tai and Spry (20) have previously demonstrated that eosinophils incubated with pronase or trypsin developed an increased capacity to form EA rosettes. In the present study, not only was the Fcγ receptor expression enhanced but mainly the cytotoxic function of eosinophils against schistosome larvae. Since a highly purified bacterial

collagenase exibited the same effect, the collagenase specificity of the schistosome enzymes is likely to be involved. This represents a potentially potent mechanism in the biology of the eosinophil, since the association of collagen, collagenase and eosinophils is common at the site of inflammation. Indeed, enzymes capable of breaking down collagen have been detected in a variety of physiological and pathological states (21). It now remains to be determined whether the collagenases act on the cells directly at their surface or through peptides resulting from the hydrolysis of their substrates.

A similar eosinophil stimulating activity was previously described with mast cell products and more particularly with ECF.A tetrapeptides (9). In view of this, the role of the mast cell as a regulator of eosinophil-mediated immunity in schistosomiasis has been suggested. In this case, mast cells, though unable by themselves to kill the parasites, would play the role of accessory cells in enhancing eosinophil anti-schistosomula cytotoxicity. Both enhancing mechanisms, by SRP or by ECF.A could act together or independently during the infection. Alternatively, depending on the infected host species, the first or the second could provide an adaptative possibility to the parasite. In the human model, for example, IgG-dependent eosinophil cytotoxicity does not require the presence of mast cells or mast cell products (18) while in the rat model the accessory cells are needed (5). In contrast, human eosinophil Fcγ receptors are stimulated by SRP (10) and therefore these parasite products could be involved <u>in situ</u> in the development of human eosinophil cytotoxicity. The similarity in eosinophil stimulating activity of the peptide ECF.A (ala-gly-ser-glu) and of secreted collagenases present in SRP pleads in favor of an indirect action by the latter. It is also necessary to define

whether the secreted proteases with collagenase activity are the same molecules at each developmental stage studied. Their characterization and their purification are in progress.

Our observations indicate that proteases present in SRP and exhibiting a collagenase specificity play a stimulatory role on the in vitro eosinophil effector function. The role of proteolytic enzymes in cellular function has been considered in the recent years. Protease inhibitors have been shown to affect such activities as cell growth (22) and the response of lymphocytes to plant lectins (23) and to antigens (24). Moreover, some lymphokines (25 - 26) and monokines (27) have been described as being or containing proteases. In our parasitic model the schistosome acts as a regulator of cellular immunity by means of secretory products, among which the proteases seem to play a key role. The parasite itself could thus control the host-parasite relationship.

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Table 1 : Increase in eosinophil activity by SRP obtained from four developmental stages of schistosomes.

SRP produced by [a]	Percentage increase in eosinophil activity [b]		
	Cytotoxicity [c]	Specific EA rosette formation [d]	Degranulation [e]
Schistosomula (day 0)	30	32	45
Lung stage schistosomula (6-day-old)	22	61	32
Liver stage schistosomula (20-day-old)	26	ND [f]	20
Adult worm (40-day-old)	30	32	35

[a] Incubation with 20 µl of SRP in each well

[b] Results are expressed in % increase of eosinophil activity after incubation with SRP in comparison to culture without SRP

[c] The effector cells consist of a mast cell-depleted eosinophil population (containing less than 1 % mast cells) after purification on metrizamide

[d] EA rosette formation was estimated by using E sensitized with rat antibody. The various cell preparations were incubated for 1 hr at 37°C. The percentage of specific rosettes was obtained by subtracting the percentage of rosettes obtained with control erythrocytes (E)

[e] The degranulation was measured microscopically after 4 hr of incubation at 37°C of the cell preparations with the different products

[f] Not done

Rat mast cell-depleted eosinophil population incubated with	Specific rosettes [a] (%)	Net change [b]
MEM	4 ± 1.5	-
Untreated SRP	44.0 ± 2.5	40 ± 4.0
Heated SRP	8.2 ± 2.0 [c]	4 ± 3.5
Untreated SRP from male schistosomes	51.0 ± 0.5	47 ± 2.0
Heated SRP from male schistosomes	16.6 ± 1.0 [d]	12 ± 1.5
Untreated SRP from female schistosomes	41.0 ± 1.0	37 ± 1.5
Heated SRP from female schistosomes	6.2 ± 1.5 [e]	2 ± 3.0
Trasylol alone (1200 KIU/ml MEM)	15.1 ± 1.0	11 ± 2.5
SRP treated with Trasylol (1200 KIU/ml SRP)	13.2 ± 0.5 [f]	9 ± 2.0

[a] All values represent the mean of three to five triplicate experiments (± SE) and were compared by Student's t test

[b] Represents the difference between the specific rosettes after incubation with MEM and with SRP

[c] Significantly inhibited when compared with untreated SRP ($p<0.0001$)

[d] Significantly inhibited when compared with non-heated male SRP ($p<0.001$)

[e] Significantly inhibited when compared with non-heated female SRP ($p<0.001$)

[f] Significantly inhibited when compared with untreated SRP ($p<0.001$)

Addition to the cytotoxicity assay	cytotoxicity (%) [a]		Net change [b]
	IRS	NRS	
MEM	9.8 ± 0.5	12.7 ± 3.0	0
Untreated SRP	48.2 ± 3.5	13.6 ± 2.0	35 ± 5.0
Heated SRP	17.0 ± 2.0 [c]	15.7 ± 0.5	2 ± 2.5
Trasylol alone (1200 KIU/ml MEM)	15.2 ± 0.5	16.4 ± 3.5	0
SRP treated with Trasylol (1200 KIU/ml SRP)	27.5 ± 1.5 [d]	14.3 ± 1.0	13.2 ± 2.5

[a] All values represent the mean of three to five triplicate experiments (± S.E) and were compared by Student's t test

[b] Represents the difference between the cytotoxicity with NRS and IRS in the same experimental conditions

[c] Significantly inhibited when compared with untreated SRP ($p < 0.0001$)

[d] Significantly inhibited when compared with untreated SRP ($p < 0.05$)

Addition to the cytotoxicity assay	% cytotoxicity [a]		Net change [b]
	IRS	NRS	
MEM	18.3 ± 7.0	6.5 ± 1	11.8 ± 7
Protease purified fractions [c]			
A1	57.6 ± 3.5	28 ± 2.0	29.6 ± 4.0 [d]
A2	56.7 ± 2.5	13.9 ± 1.5	42.8 ± 3.0 [e]
A3	23.9 ± 4.5	16.3 ± 2.0	7.6 ± 5.0
B1	39.5 ± 2.0	23 ± 3.5	16.5 ± 4.0
B2	29 ± 4.0	17 ± 5.0	12 ± 6.5
B3	46.9 ± 1.5	3.8 ± 2.5	43.1 ± 3.0 [f]

[a] All values represent the means of two triplicate experiments and were compared with Student's t test

[b] Net change represents the difference between the cytotoxicity with NRS and IRS in the same experimental conditions. The standard deviation was calculated from S.D IRS and NRS

[c] A represents the fractions of proteases able to cleave the Z-gly-pro-leu-gly-pro substrate and B the fractions of proteases able to cleave Azocoll after both preparative electric focusing and filtration through ACA 34 gel

[d,e,f] Significantly different when compared to medium (d : $p < 0.01$; e : $p < 0.001$; f : $p < 0.001$)

schistosomula *in vitro*.

Addition to the cytotoxicity assay	Cytotoxicity % [a]		Net change [b]
	IRS	NRS	
MEM	18.4 ± 9.0	5.1 ± 2.0	13 ± 10.5
Collagenase 100 µg/ml	52.7 ± 5.5 [c]	3.6 ± 1.5	49 ± 7.0
Collagenase 500 µg/ml	12.5 ± 2.5	2.9 ± 2.0	9 ± 4.5

[a] All values represent the mean of three triplicate experiments (± S.E) and were compared by a Student's *t* test

[b] Represents the difference between the cytotoxicity with NRS and IRS in the same experimental conditions

[c] Significantly different when compared to medium ($p < 0.001$).

LEGENDS OF THE FIGURES

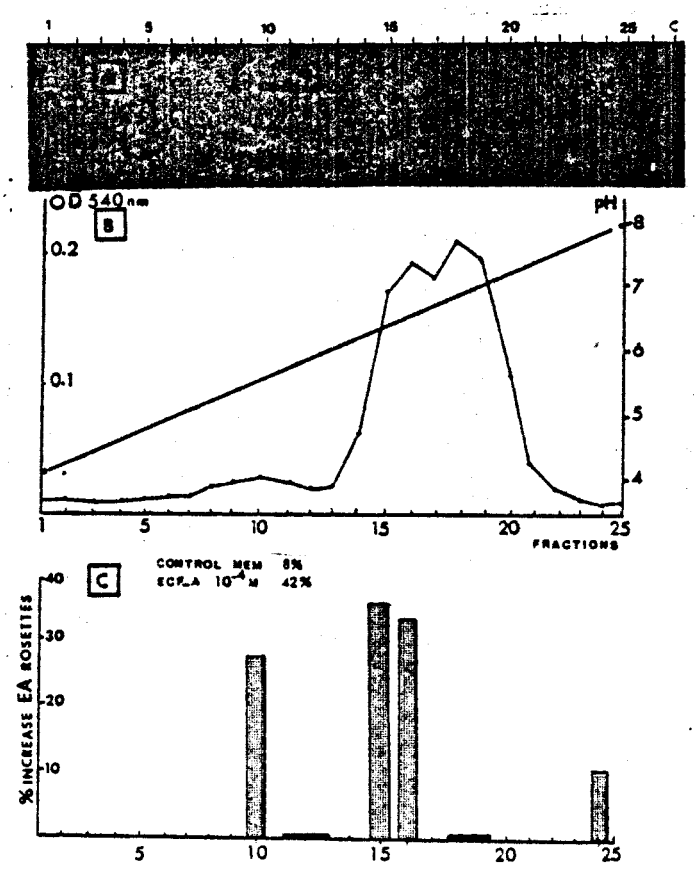
Figure 1 : Preparative isoelectric focusing of adult extracts over the pH range 3.5 to 10. Fractions were assayed for Z-gly-pro-leu-gly-pro hydrolytic activity (A) after chromatography in BAW on Whatman N°3 paper for azocollytic activity for absorbance at 540 nm (B) and for specific enhancement of eosinophil EA rosette formation (C).

Figure 2 : Hydrolysis of Azocoll and Z-gly-pro-leu-gly-pro after filtration through ACA 34 Ultrogel (90 x 2 cm column). Separation into 6 distinct fractions. For Z-gly-pro-leu-gly-pro, results were estimated and graded according to the degree of coloration of the spot from (-) : non hydrolysed to (+++) : strongly hydrolysed.

Eosinophil EA rosette formation (B) and IgG dependent eosinophil anti-schistosomula cytotoxicity (C) have been tested with a solution of 2 µg/ml of total proteins of each fraction (▒▒▒, Schistosomula incubated in the presence of IRS + the parasite extract ; ▬▬, Schistosomula incubated in the presence of NRS + the parasite extract).

Figure 3 : Preparative isoelectric focusing of adult worm extract over the pH range 3.5 to 10. Fractions were assayed for azocollytic activity by absorbance at 540 nm (B) and for Z-gly-pro-leu-gly-pro hydrolytic activity (A) after chromatography in BAW on Whatman N°3 paper (see Materials and Methods). Results were estimated and graded from (-) : non hydrolysed to (+) : strongly hydrolysed. The two fractions F.A and F.B were then separately purified by gel filtration.

Figure 4 : Filtration through ACA-34 Ultrogel (90 x 2 cm column) of the fractions F.A and F.B corresponding to parasite proteases partially purified by preparative isoelectric focusing (see Fig. 3). Fractions A1, A2 and A3 hydrolysed the Z-gly-pro-leu-gly-pro substrate (A) ; fractions B1, B2 and B3 exhibited an azocollytic activity monitored at 540 nm.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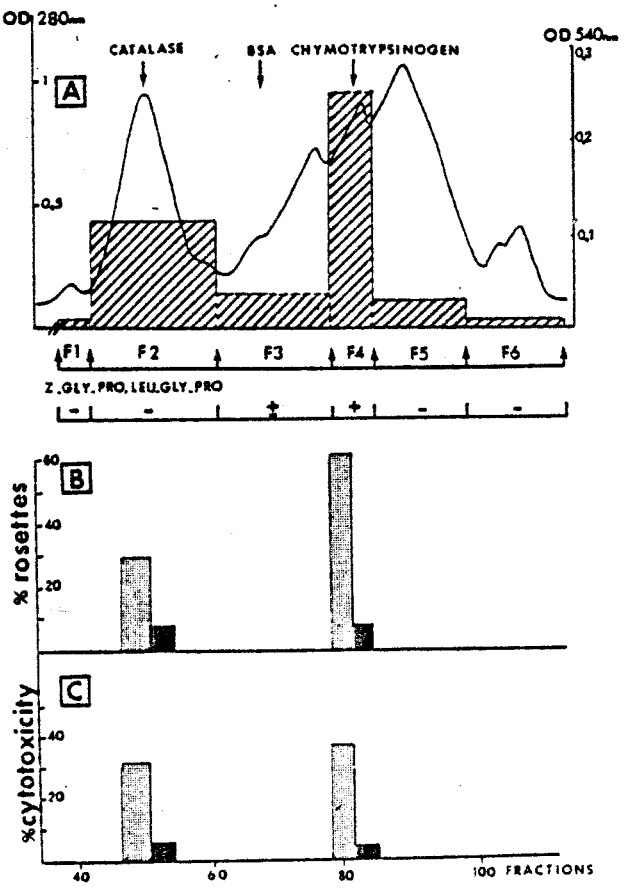
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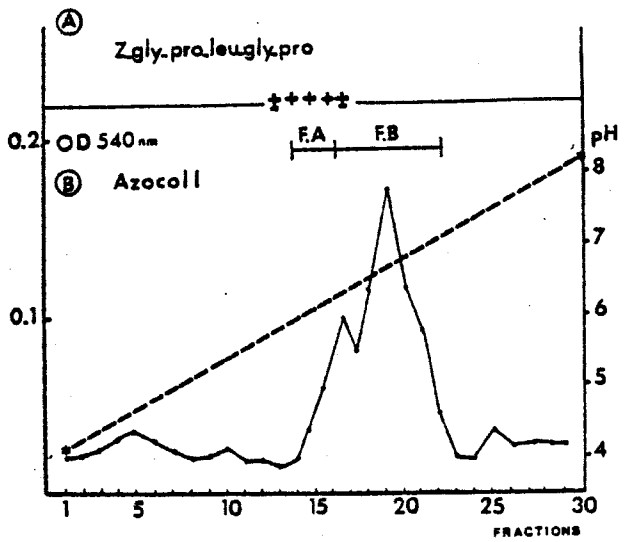
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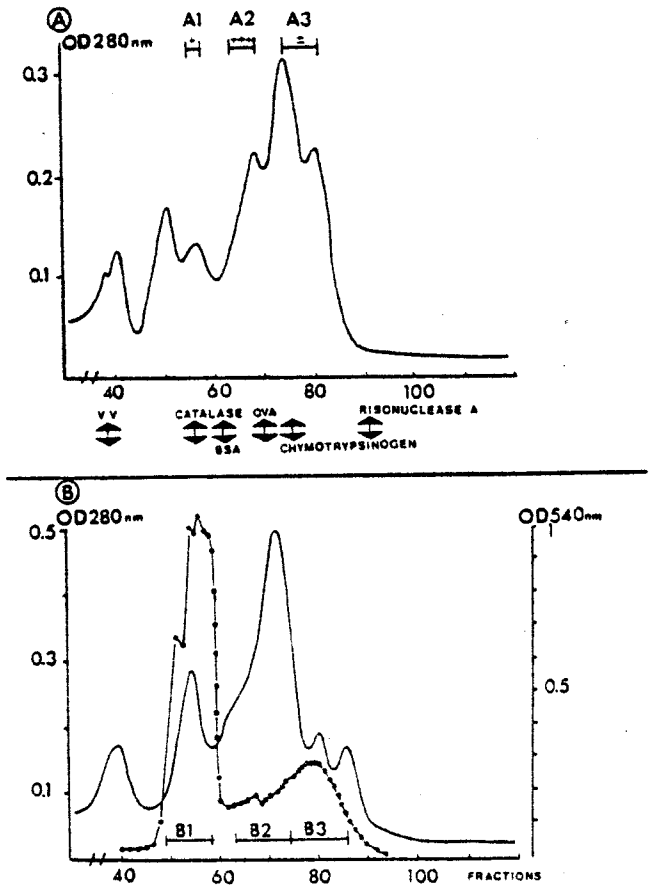
FRACTIONS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ARTICLE n° 8 : ACTIVITES DES PROTEASES NEUTRES A DIFFERENTS STADES DE DEVELOPPEMENT DE S. MANSONI CHEZ SON HOTE MAMMIFERE.

L'implication des protéases neutres dans certains mécanismes de régulation de la réponse cellulaire dirigée contre le parasite nous a conduit à inventorier ces activités protéolytiques. Cet article a été consacré à une étude comparative des protéases neutres.

1) Sur trois stades d'évolution des schistosomes chez l'hôte mammifère (le hamster dans cette étude) que sont les schistosomules (JO), les jeunes schistosomes de 20 jours retrouvés dans le foie (J20) et les vers adultes (J40).

2) Dans des homogénats totaux ou après extraction à la saponine-calcium afin de différencier les protéases associées aux téguments parasitaires des protéases totales.

Pour cette étude deux substrats ont été plus particulièrement utilisés : l'azocaseïne afin de détecter les endoprotéases et la leucine-p-nitroanilide afin d'étudier les amino-peptidases. La recherche de carboxypeptidases à l'aide de 4 substrats NH_2 substitués ne nous a pas permis de détecter d'activités quantifiables.

Ces résultats mettent en évidence que plusieurs activités distinctes peuvent être retrouvées dans des extraits totaux et saponine-calcium chez les schistosomes. Par ailleurs, par l'utilisation d'inhibiteurs spécifiques, on peut mettre en évidence que l'activité endoprotéase majeure des schistosomules est une

sérine protéase tandis que les stades ultérieurs se caractérisent par des activités thiol-protéases. Par contre, les aminopeptidases présentent les mêmes caractéristiques quels que soient les stades de l'extrait étudié. Nous confirmons que cette activité aminopeptidase majeure est une métallo-peptidase puisqu'elle est inhibée par l'O-phenantroline.

L'étude biochimique par des méthodes préparatives classiques comme le gel filtration sur ACA 34 et l'électrofocalisation mettent en évidence au moins cinq activités caséolytiques dont la principale a un pHi de 4,4 et quatre activités azocollytiques dont deux majeures se localisent au niveau des pH 6.5 et 6.8. Les trois aminopeptidases mises en évidence par l'hydrolyse des peptides synthétiques comme la leucine-p-nitroanilide ou le dipeptide Leu-Gly sont associées au moins pour deux d'entre elles à des activités iminopeptidases détectées par l'hydrolyse du dipeptide Pro-Leu.

Cette étude quoique partielle montre la variété des protéases présentes chez le schistosome. Ceci corrobore les études précédentes mettant en évidence l'effet régulateur de certaines d'entre elles. C'est en effet la spécificité de ces protéases parasitaires qui sont à l'origine des effets modulateurs étudiés.

Article N 8

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NEUTRAL PROTEASE ACTIVITIES AT DIFFERENT DEVELOPMENTAL STAGES OF *SCHISTOSOMA MANSONI* IN MAMMALIAN HOSTS

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Abstract—Neutral protease activities of schistosomula, 20 day old and adult worms of *S. mansoni* have been studied.

1. The neutral enzymes of the three development stages are able to hydrolyse numerous natural and synthetic substrates.
2. The enzymes of homogenates and saponin $CaCl_2$ extracts show significant discrepancies in their specificities, optimum pH activities and in the effects of enzyme inhibitors.
3. A serine protease activity was specific for the schistosomula stage whereas thiol proteinases characterize the later stages of evolution. A metalloaminopeptidase activity was shown at all three stages.
4. Gel filtration chromatography and isoelectric focusing of an adult worm homogenate demonstrated the presence of at least 5 caseolytic and 4 azocollytic neutral activities. Three aminopeptidases, one of which exhibited iminopeptidase specificity were revealed by the same procedure.

Since the neutral proteolytic enzymes appear to play a major role in the host parasite relationship a study of these enzymes was carried out. Indeed it has been previously demonstrated that after binding onto the schistosomulum surface by the Fc portion, IgG was hydrolysed by secreted neutral proteases (Auriault *et al.*, 1980). After cleavage, a small IgG fragment bearing Fc determinants remained on the larval surface and has been shown to be involved in the activation of the classical pathway of complement by schistosomula (Ouassi *et al.*, 1981). In addition, the small peptides liberated into the medium by this proteolytic cleavage exerted a significant inhibition of macrophage activity, especially the antibody dependent cellular cytotoxicity directed against the schistosomula (Auriault *et al.*, 1980; Auriault *et al.*, 1981).

Some schistosoma proteolytic enzymes have already been studied in cercariae, adult worms or eggs. In cercariae proteases contained in preacetabular glands have been characterized and purified (Gassinelli *et al.*, 1966; Dresden & Asch, 1972; Stirewalt, 1978). The results obtained indicated the presence of multiple proteolytic activities able to hydrolase numerous substrates. However, it is clear that the major activity seems to exhibit a chymotryptic like specificity characterized in a crude extract using specific synthetic substrates and inhibitors (Gazzinelli *et al.*, 1966) or after purification (Landsperger *et al.*, 1981, in press).

The main proteolytic enzyme described in adult worms is a thiol acidic hemoglobinase (Grant & Senft & Senft, 1971; Deelder *et al.*, 1977; Dresden & Deelder, 1979). This enzyme, present in worm vomitus, was shown to be antigenic in mice (Deelder *et al.*, 1977) or able to induce a rapid histaminic skin response in various animals including man (Senft *et al.*, 1979). An adult azocollytic activity found in the homogenate and protease rich-saponin $CaCl_2$ extracts of *S. mansoni* has been described by Cesari *et al.* (1981).

In eggs a collagenase like activity has been demonstrated using azocoll (Kloetzel, 1968; Asch & Dresden, 1979) BSA or rat tail collagen (Smith, 1971) as substrates.

In order to reach a better understanding of the possible role of these enzymes in the host parasite relationship, the present investigation was undertaken to characterize further the neutral proteolytic enzymes of *Schistosoma mansoni* during its development in a mammalian host.

MATERIALS AND METHODS

Reagents

Substrates and inhibitors were purchased from Sigma chemical Co., St Louis, USA.

ACA 34 ultrogel was provided by Pharamindustrie, France.

Ampholines were purchased from LKB Bromma Sweden.

Saponin was purchased from Merck, Darmstadt, Germany.

Parasite life cycle and preparation of schistosomula

A Puerto Rican strain of *Schistosoma mansoni* was used throughout the study (Capron *et al.*, 1974). Schistosomula were prepared *in vitro* from cercariae by the mechanical procedure described by Ramalho-Pinto *et al.* (1974). Before homogenization or saponin–$CaCl_2$ treatment the schistosomula were incubated for 4 hr at 37°C in Eagles minimum medium (MEM, Difco, Detroit, MI.) and washed 6 times. After the last washing schistosomula were incubated for

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16 hr at 37°C in MEM to remove most of the cercarial enzymes. The absence of bacterial contamination was controlled at the end of incubation together with the viability of schistosomula which was always superior to 95%. After centrifugation at 150 *g* for two minutes the pellet containing schistosomula or the supernatant called schistosomula released products (SRP) was recovered and then treated.

Recovery of 20 day old and adult worms

The 20 day old larvae and the adult worms were collected by perfusion of the liver and the mesenteric veins respectively 20 or 40 days after infestation of hamsters (1000 cercariae per animal). The living parasites were then extensively washed at least six times in Hank's balanced salt solution for three hours.

Homogenization and saponin–$CaCl_2$ treatment

The parasites were first treated by sonication (microsonde, 3 times 5 sec, 70 watts sonifier B-12, Branson sonic) at 0°C in 0.1 M phosphate buffer pH 7.2. This treatment destabilized the membrane and was followed by homogenization using a hand rotated Potter–Elvehjem homogenizer with a tight fitting teflon pestle. The temperature was maintained at 4°C during this treatment. The homogenates were then centrifuged at 20 000 *g* for 1 hr at 4°C. The lipids were removed from the surface and the supernatant was called the crude enzymatic extract.

The saponin extraction was carried out in some experiments to define some of the protease activities associated with the surface of the parasites. One hundred adult worms, 800 20 day old larvae or 80,000 schistosomula (corresponding to 1.7 to 1.8 mg of total protein) were washed several times in 0.85% NaCl. After the last centrifugation, the supernatant was removed and replaced by a solution of 0.5% saponin in 3% $CaCl_2$ and 0.85% NaCl (120 μl in these conditions). After 5 min at room temperature, the supernatants were carefully removed and centrifuged at 20 000 *g* for 2 hr at 4°C. The supernatants were fractionated through a Sephadex G-50 column (0.9 × 30 cm) in 0.1 M phosphate buffer pH 7.3 to eliminate the saponin and undesired ions.

The protein content of enzyme preparations was determined by the method of Lowry *et al.* (1951).

Enzyme assays

The arylamide substrates were solubilized in methanol (6 mg/ml) and diluted 1:10 in sterile 0.1 M phosphate buffer pH 7.2 containing 0.01% sodium azide. The synthetic dipeptides were solubilized at 1 mg/ml, azocaseine and azocoll were used 1 and 2% respectively in the same buffer. In some experiments the substrates were solubilized in 0.2 M acetate buffer pH 4 or 5.5, or 0.2 M amenediol pH 9.

The enzyme inhibitors were used at concentrations of 10^{-3} or 10^{-4} M.

With LNA. (Leucine-*p*-nitroanilide) and other arylamide substrates, 1 ml of substrate and 20 μl of parasite extract were incubated at 37°C. The yellow colouration indicating the hydrolysis was measured at 405 nm (Beckman DB spectrophotometer). A blank was used each time with 20 μl of parasitic extract in 1 ml of substrate, the hydrolysis of which was rapidly stopped by adding a drop of concentrated acetic acid (30% by vol.). After 1–6 hr according to the experiments the hydrolyses were stopped in the same manner.

Azocasein and azocoll. 2 ml of each substrate and 50 μl of parasite extract were incubated at 37°C for 6–10 hr under agitation. The hydrolysis of azocoll was measured directly at 540 nm. At the end of the hydrolysis of the azocasein, the hydrolysate was precipitated with 6% TCA (final volume) and filtered through Whatman paper No. 542. The filtrate was diluted 1:1 in 0.5 M NaOH and left for 1 hr at room temperature. The colouration was then measured at 440 nm. A blank was used each time with 50 μl of parasite extract in 2 ml of substrate rapidly precipitated with TCA.

Each experiment and each hydrolysis was repeated at least twice and the results of representative experiments are given.

Gel filtration chromatography

In order to separate the different protease activities according to their molecular weights the homogenate of adult worms (20 mg of total proteins) was filtered through a column (2 × 90 cm) containing ACA 34 Ultrogel (Pharmindustrie France). The absorbance of the eluate was monitored at 280 nm by an LKB Uvicord S (Bromma, Sweden).

Isoelectric focusing

Preparative horizontal bed isoelectric focusing was carried out using an LKB multiphor apparatus. Focusing of the adult worm extract (20 mg of total proteins) was performed in a 100 ml bed of Sephadex G-75 containing 5% carrier ampholines, pH range 3.5–10 for 16 hr at 8 watts constant power. The gel bed was then divided into 30 sections using an LKB template. Each gel fraction was eluted in 10 ml syringe barrels fitted with a porous plastic sinter with 3 ml water. After direct pH determination the eluted fractions were then dialysed overnight against phosphate buffer 0.1 M pH 7.0 containing 0.15 M NaCl to remove ampholines.

Paper chromatography

Unidirectional paper chromatography was used to investigate the hydrolysis of 50 μg of dipeptide or *CBZ*-Gly-Pro-Leu-Gly-Pro substrates by 10 or 20 μl of parasite extracts. The hydrolysates were spotted on Whatman paper no. 3 (46 × 57 cm). Controls with parasite extracts and substrates alone were performed. Elution was carried out for 10 hr at room temperature in butanol:acetic acid:water 4:1:5 (BAW). The dried spots were revealed using a 1% ninhydrin solution in acetone.

RESULTS AND DISCUSSION

Neutral activities of parasite extracts

The proteolytic and peptidasic activities of schistosomula, 20 day old worms and adult worms were tested at pH 7.2 and 37°C and compared using proteins, mono- or di-substituted peptides or dipeptides as substrates (Table 1). There were only small discrepancies between the specificities of the three enzymatic extracts but the results showed the diversity of the neutral enzymatic material of *Schistosoma mansoni* given the variety of the different substrates hydrolysed. In contrast, the *N*-substituted CBZ-peptides used were never hydrolysed by the extracts. This suggests that the observed hydrolyses were not due to carboxypeptidase activity.

The eosinophil chemotactic factor of anaphylaxis (ECF.A), Ala-Gly-Ser-Glu or the analogues Val-Gly-Ser-Glu and Val-Pro-Ser-Glu were hydrolysed by both homogenate (Table 1) or incubation products (Fig. 1) of schistosomula. This indicates that schistosomula possess and secrete proteinases able to hydrolyse ECF.A which has been shown to be one of the major regulators of the *in vitro* antibody dependent eosinophil cytoxicity directed against the larvae (Capron M. *et al.*, 1978; Capron M. *et al.*, 1981). Many unsubstituted dipeptides, the majority of which possessed leucine residues in the N or C-terminal position were hydrolysed, even Pro-Leu and Pro-Gly. This suggests a wide spectrum of the aminopeptidase activities and the presence of imino-peptidase or pro-

Table 1. Hydrolysis at pH 7.2 of different substrates by homogenates of *Schistosoma mansoni* at three stages of evolution in a mammalian host. The degree of hydrolysis was estimated and graded from ± (weakly hydrolysed) to + + + (strongly hydrolysed)

Substrates	Schistosomula	20 day old worms	Adults
Proteins or disubstituted synthetic peptides			
Glut-L Phe- L Ala 4 NA	—	—	—
Benz L Arg 4 NA	(±)	—	—
Azocaseine	+	+	+
Azocoll	+	+	+
IgG	+	ND	ND
Monosubstituted peptides			
N-CBZ-Gly-Leu	−	−	−
N-CBZ-Ala-Ala	−	−	−
N-CBZ-Glu-Phe	−	−	−
N-CBZ-Leu-Tyr	−	−	−
Leu-*p*-nitroanilide	+ + +	+ + +	+ + +
Ala-*p*-nitroanilide	+ + +	+ + +	+ + +
Lys-*p*-nitroanilide	+ +	+	ND
Pro-*p*-nitroanilide	(±)	+	+
Tyr *p*-nitroanilide	(±)	+ +	ND
CBZ-Gly-Pro-Leu-Gly-Pro	+	+	+
Unsubstituted peptides			
Leu-Leu	+ + +	+ + +	+ + +
Leu-Ser	(±)	+ + +	+ + +
Ser-Leu	−	+ +	+
Leu-trp	+ +	+ +	+
Trp-Leu	+ +	+ +	+ +
Leu-Pro	+	ND	−
Pro-Leu	+ +	+ + +	+ + +
Ala-Leu	+	+ + +	+ + +
Ala-Val	−	+ + +	+ + +
Val-Ala	ND	(±)	(±)
His-Leu	+ +	(±)	(±)
Lys-Leu	+ +	+	+
Met-Leu	+ +	(±)	(±)
Gly-Pro	−	+	+
Pro-Gly	MD	+ +	+ + +
Leu-Gly	(±)	+ +	+ +
ECF-A			
Ala-Gly-Ser-Glu	+	ND	ND
Val-Gly-Ser-Glu	+	ND	ND
Val-Pro-Ser-Glu	+	ND	ND

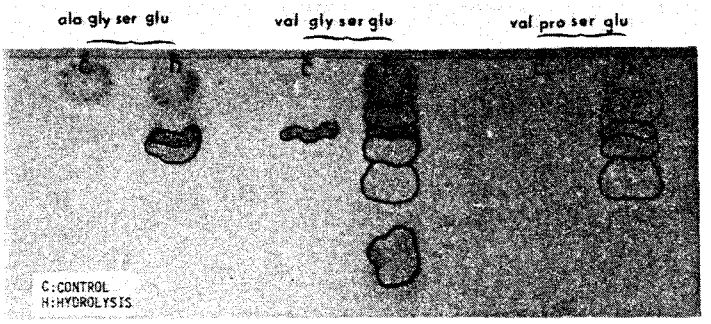

Fig. 1. Cleavage of ECF. A tetrapeptides (50 μg) and two synthetic analogues by 50 μl of incubation products of 20,000 schistosomula in 1.5 ml of PBS (+2% glucose). The hydrolysis was carried out at 37°C overnight and chromatographed on Whatman No. 3 paper and revealed by a 1% ninhydrin–acetone solution. Each spot not corresponding to the control represents a smaller peptide than ECF.A or an aminoacid. The control was ECF.A alone incubated overnight at 37°C and chromatographed as for the hydrolysate. Incubation products incubated alone presented no coloured spots. This experiment was repeated twice with the same results.

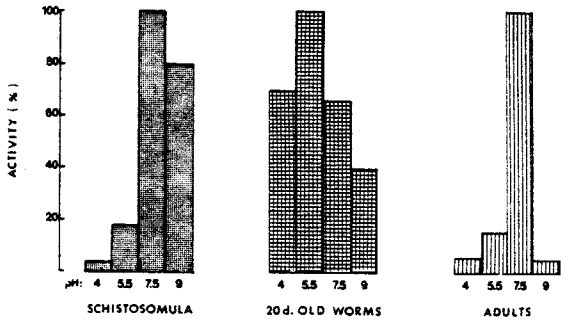

Fig. 2. pH effect on proteolytic activities of homogenates of *Schistosoma mansoni* at three stages of evolution using 1% azocasein as substrate.

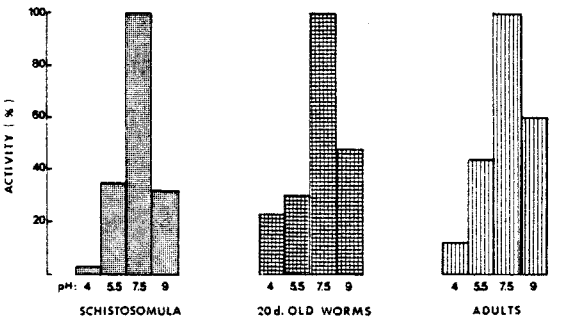

Fig. 5. pH effect on peptidase activities of saponin–$CaCl_2$ extracts of *Schistosoma mansoni* at three stages of evolution using LNA as substrate.

linase activities. This is corroborated by the hydrolysis of the monosubstituted peptide Z-Gly-Pro-Leu-Gly-Pro. Indeed this hydrolysis observed with extracts of the 3 development stages studied, is characterized by the liberation of free leucine detectable in descending paper chromatography (see below).

Effect of pH on proteolytic and peptidasic activities of homogenates and saponin–$CaCl_2$ extracts at three stages of development

The different parasite extracts prepared in 0.1 M phosphate buffer pH 7.2 either after homogenization or after saponin–$CaCl_2$ treatment were tested using 1% azocasein and LNA as substrates at pH 4, 5.5, 7.5 and 9 at 37°C.

The endoprotease activities shown after hydrolysis of azocasein exhibit different optimum pH activities according to the development stage in the mammalian host and the extraction procedure. Indeed both schistosomula homogenates or saponin–$CaCl_2$ extracts evidenced optimum neutral and basic activities (pH 7.5) whereas 20 day old and adult worm saponin $CaCl_2$ extracts and homogenates exhibited activities distinguished by their optimum pH (Fig. 2 and 3). These results indicate that first, the protease activities of the two extracts differ by their optimum

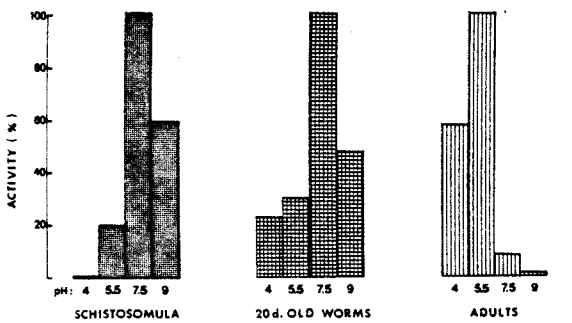

Fig. 3. pH effect on proteolytic activities of saponin–$CaCl_2$ extracts of *Schistosoma mansoni* at three stages of evolution using 1% azocasein as substrate.

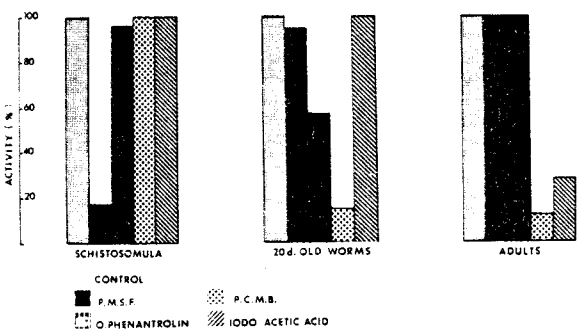

Fig. 6. Effect of four inhibitors on protease activities of homogenates of *Schistosoma mansoni* at three stages of evolution using 1% azocasein in 0.1 M phosphate, pH 7.2 as substrate.

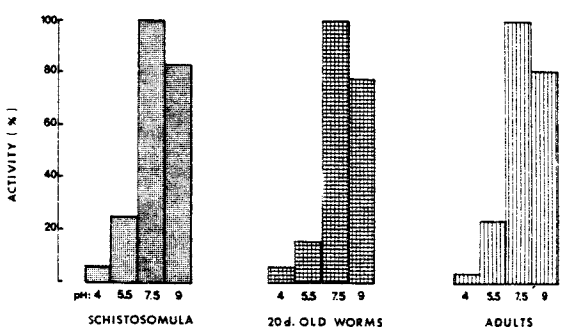

Fig. 4. pH effect on peptidasic activities of homogenates of *Schistosoma mansoni* at three stages of evolution using LNA as substrate.

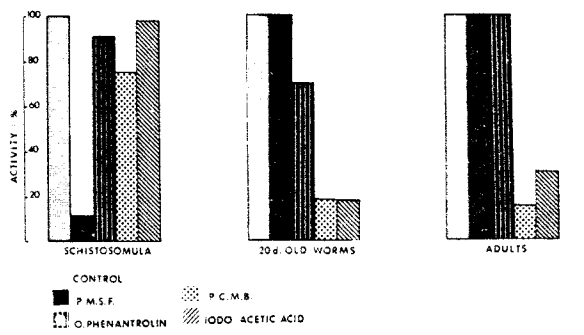

Fig. 7. Effect of four inhibitors on protease activities of saponin–$CaCl_2$ extracts of *Schistosoma mansoni* at three stages of evolution using 1% azocasein in 0.1 M phosphate pH 7.2 as substrate.

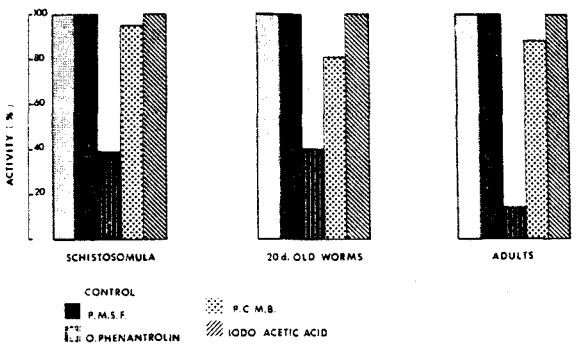

Fig. 8. Effect of four inhibitors on peptidase activities of homogenates of *Schistosoma mansoni* at three stages of evolution using LNA in 0.1 M phosphate pH 7.2 as substrate.

pH in 20 day old and adult worms and second that they also differ according to the stage of ontogenesis. This points out the likely diversity of the proteases extracted at neutral pH in *Schistosoma mansoni*. Such a diversity was not found for the leucine aminopeptidase activities (Figs 4 and 5). Indeed whatever the extract or the development stage examined the optimum pH was 7.5 with 30 to 80% of the activity remaining at pH 9. The saponin–$CaCl_2$ extracts probably select most of the activities linked to the tegument of the parasite and the comparison with the homogenates reveal interesting differences which were verified using enzymatic inhibitors.

Effect of inhibitors on proteolytic and peptidasic activities of homogenates and saponin $CaCl_2$ extracts at three stages of development

In order to define the comparative approach between the homogenates and the saponin $CaCl_2$ extracts at the three stages of evolution we tested the protease and the aminopeptidase activities in the presence of four inhibitors: phenyl-methyl sulfonyl fluoride (PMSF), parachloromercuribenzoate (PCMB), *O*-phenanthroline and iodoacetic acid used at a final concentration of 1×10^{-3} M. All the hydrolyses were carried out at 37°C in 0.1 M phosphate buffer pH 7.2 which was the buffer used for the extraction. Both schistosomula homogenate and saponin–$CaCl_2$ extracts were strongly inhibited by PMSF suggesting the presence of a major serine protease activity expressed at this larval stage (Figs 6 and 7). Such a secreted serine protease activity able to cleave IgG which is bound to the schistosomula surface receptor has been previously described (Auriault *et al.*, 1981). The results obtained with extracts of the two other stages are distinct from those obtained with schistosomula since PMSF never inhibited the activities present in homogenates or saponin–$CaCl_2$ extracts of 20 day old and adult worms. In contrast, PCMB inhibited all the protease activities indicating the replacement of serine proteases by thiol proteases concomitant with the parasite evolution. To confirm this hypothesis iodoacetic acid also inhibited the protease activities of the older development stages except those expressed in the 20 day old worm homogenate. On the other hand a partial inhibition with *O*- phenanthroline can be observed in the two extracts obtained from the 20 day old worms.

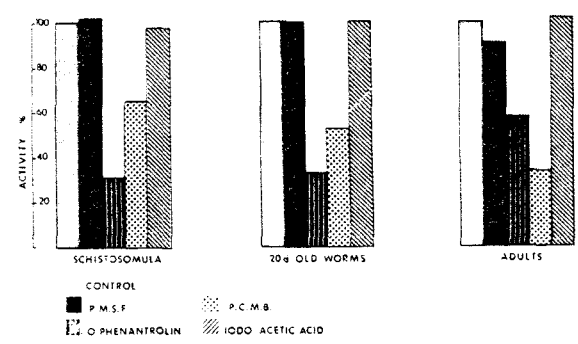

Fig. 9. Effect of four inhibitors on peptidase activities of saponin–$CaCl_2$ extracts of *Schistosoma mansoni* at three stages of evolution using LNA in 0.1 M phosphate pH 7.2 as substrate.

The aminopeptidase activities of the homogenates and saponin $CaCl_2$ extracts of the three development stages were inhibited by both *O*-phenanthroline and PCMB (Fig. 8 and 9). The major aminopeptidase activities at the three stages are probably due to metalloaminopeptidases (already described by Cesari *et al.*, in press). The partial inhibition by PCMB could indicate that the active conformation of some of the enzymatic proteins might involve sulphydryl residues. The presence of these activities in the saponin-$CaCl_2$ extract, the homogeneity of the optimum pH activities and the similarities of the effects of inhibitors at all the development stages suggests that some of these aminopeptidases are associated with the tegument during the growth of the parasite in the mammalian host.

Gel filtration chromatography of the neutral adult proteinase activities

In an attempt to characterize the neutral adult proteolytic enzymes, a concentrated homogenate was subjected to Ultrogel ACA-34 chromatography. Six column fractions were assayed for proteolytic and peptidasic activities against LNS, azocasein, azocoll, Pro-Leu-Gly and Z-Gly-Pro-Leu-Gly-Pro substrates. The results of assays using these substrates are shown in Fig. 10. They indicate the presence of several protease and aminopeptidase activities separated by their molecular weights. It is clear that the individual column fractions display quantitatively and probably qualitatively different proteolytic activities. Two separated peaks exhibit a proteolytic activity (F2 and F4) using azocoll and azocasein as substrates. The first corresponds to high molecular weight molecules (superior to 230,000) the other to molecules of a molecular weight of about 65,000. The aminopeptidase activities are located in three peaks (F2 and mainly F3 and F4) corresponding to a large spectrum of molecular weight. The iminopeptidase and endopeptidase activities able to hydrolyse respectively Pro-Leu and Z-Gly-Pro-Leu-Gly-Pro substrates are present in the same peaks as those hydrolysing aminopeptidase substrates (LNA, Leu-Gly). Further purification is required to define the number of neutral proteolytic and peptidasic species present in adult extracts.

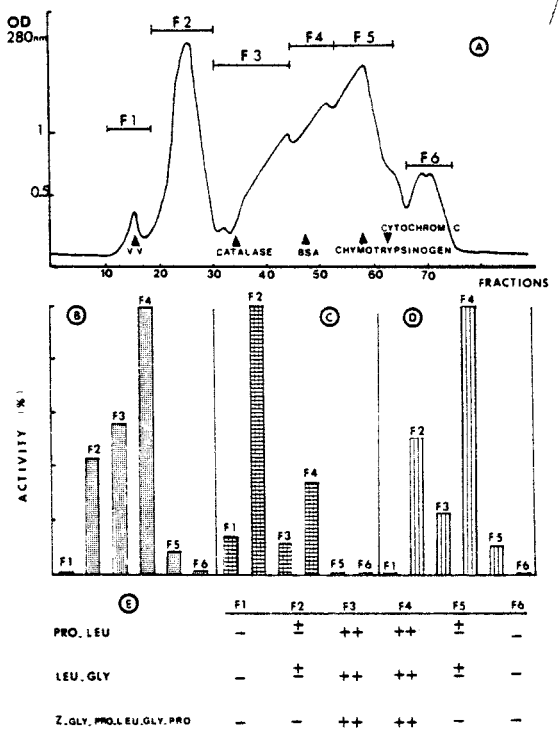

Fig. 10. Hydrolysis at 37°C of azocasein, azocoll, LNA, Pro-Leu, Leu-Gly and *Z*-Gly-Pro-Leu-Gly-Pro in 0.1 M phosphate, pH 7.2 by fractions of a homogenate of adult worms after filtration through ACA-34 Ultrogel (column 90 × 2 cm). A. profile of the elution monitored as the optical density at 280 nm. Separation into six distinct fractions. B. hydrolysis of LNA by 20 μl of each fraction for 3 hr. C. hydrolysis of 1% azocasein by 50 μl of each fraction 10 hr. D. hydrolysis of 2% azocoll by 50 μl of each fraction. BCD are expressed in % of maximal activity. E. hydrolysis of Pro-Leu, Leu-Gly, *Z*-Gly-Pro-Leu-Gly-Pro (40 μg for each substrate) by 20 μl of each fraction and followed by a paper chromatography in BAW (10 hr) revealed by a 1% ninhydrin–acetone solution. Results are estimated and graded in degree of coloration of the spots from −(non hydrolysed) to + + (strongly hydrolysed).

Preparative isoelectric focusing of the neutral adult proteinase activities

The neutral proteinase activities of homogenates from adult worms against a variety of substrates have been tested after isoelectric focusing (pH range 3–10). The optical density profile at 280 nm is shown in Fig. 11. The majority of the proteins of the extract had pHi's between 5.5 and 7.5. The activities of neutral proteases able to hydrolyse azocasein are divided into five peaks with isoelectric points at pH 4.4, 5.2, 6, 6.8 and 7.4 (Fig. 12). The aminopeptidase activities evidenced after hydrolysis of LNA are represented by a major peak with an isoelectric point at pHi 5.7 and two minor peaks at 6.8 and 7.4. If the major peak is distinct from the protease activities, the latter two seem to be associated with them. This could indicate either two enzyme molecules possessing the same pHi or that both specificities are borne by the same proteinase. The latter is more probable. This can be related to the results described above showing that the major protease and peptidase activities are distinguishable by the effects of the inhibitors. The major aminopeptidase probably also exhibits an iminopeptidase activity (Fig. 3; Table 2) since both are not only found at the same isoelectric point (5.7) but also share the same pH optimum of activity and the same effects of inhibitors.

This iminopeptidase activity cleaving Pro-Leu is also present at pHi 6.8 and 7.4 associated with slight protease and aminopeptidase activities although the activity at pHi 7.4 strongly hydrolysed Leu-Gly (Fig. 13).

Z-Gly-Pro-Leu-Gly-Pro and Azocoll, considered as collagenase substrates were also tested with the same fractions. The first was hydrolysed by an activity with an isoelectric point of 5.4 to 5.6 (Fig. 13). Isoelectric focusing resolved azocollytic activities into two major (pHi 6.5 and 6.8) and two minor peaks (pHi 5.2 and 7.4) which could not be related to the endopeptidase activity able to cleave *Z*-Gly-Pro-Leu-Gly-Pro. This does not signify that such molecules are true collagenases but that they exhibit some collagenase specificities. In regard to these results the relationship between the activities able to cleave azocaseine, azocoll and *Z*-Gly-Pro-Leu-Gly-Pro is still not clear. In contrast, the isoelectric points, as for the effects of the inhibitors (especially *O*-phenantrolin) clearly indicate that the major aminopeptidase is not related to the endopeptidase activity hydrolysing *Z*-Gly-Pro-Leu-Gly-Pro (Table 2). Moreover, the need for several enzymes to hydrolyse this last substrate is not excluded.

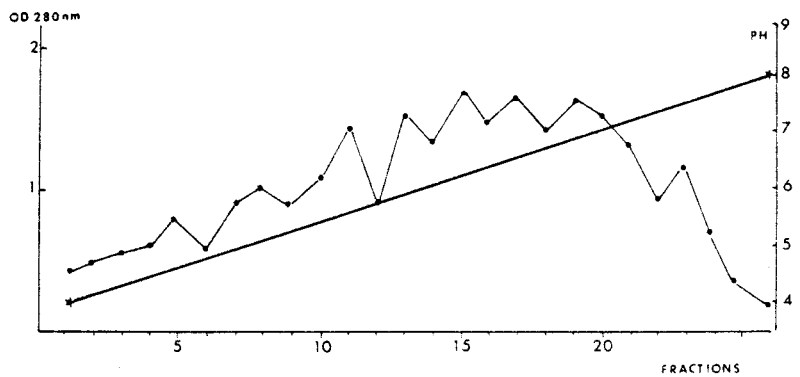

Fig. 11. Preparative isoelectric focusing of adult homogenates over the pH range 3–10. Parasitic extract (20 mg) was applied and electrofocused for 16 hr at 4°C. Fractions were assayed by absorbance at 280 nm. The pH gradient was determined as described in Materials and Methods.

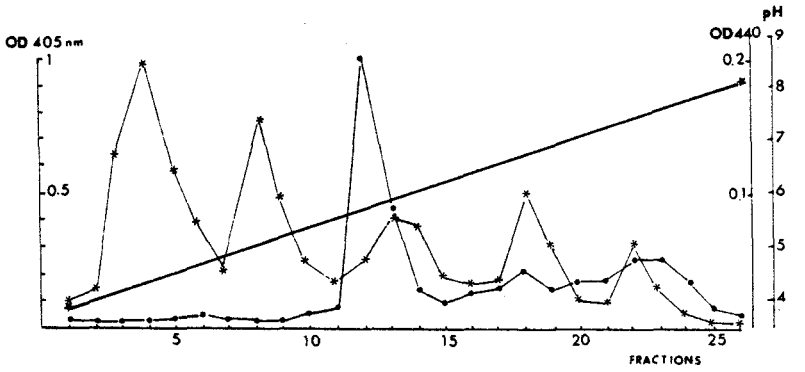

Fig. 12. Preparative ioselectric focusing of adult homogenate over the pH range 3 to 10. Fractions were assayed for azocasein activity *——* by absorbance at 440 nm (see Materials and Methods) or for LNA activity O——O at 405 nm.

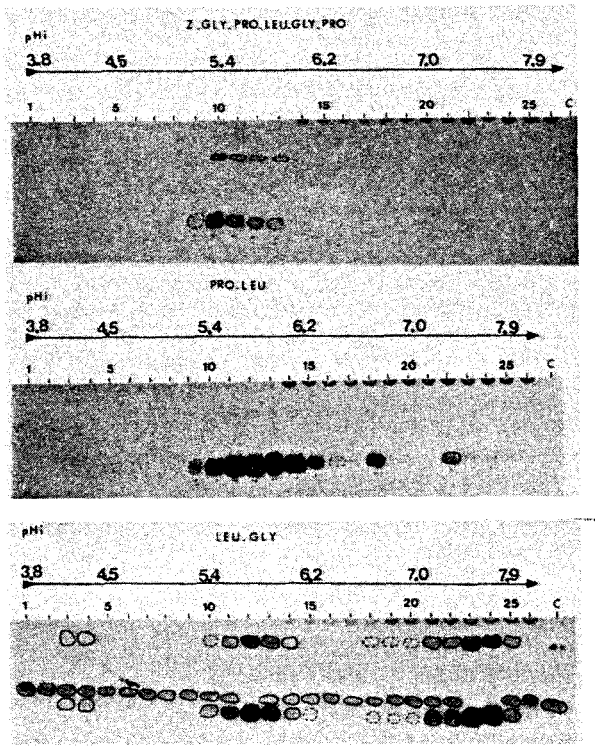

Fig. 13. Preparative isoelectric focusing of adult homogenate over the pH range 3–10. Fractions were assayed for Z-Gly-Pro-Leu-Gly-Pro, Pro-Leu and Leu-Gly activity. After hydrolysis of each fraction the hydrolysates were chromatographed in BAW on Whatman No. 3 paper for 10 hr and revealed by a 1% ninhydrin solution in acetone.

CONCLUSION

This study was conducted to define further the neutral *Schistosoma mansoni* proteinases and should be considered as the first step of a more general investigation. Results presented here using a variety of substrates and inhibitors suggest that several distinct proteases are found in both homogenates and saponin $CaCl_2$ extracts at three development stages of the parasite. The schistosomula exhibit a major serine protease activity while the 20 day old and adult worms possess thiol proteases differing by their pH optimum of activity. In contrast, the aminopeptidases exhibit the same characteristics whatever the extraction procedure or the stage. As *O*-phenanthroline is the main inhibitor the major activity is probably a metallopeptidase. The purification by ACA-34 gel chromatography of a crude extract of adult worms confirms the heterogeneity of the neutral proteases using azocasein, azocoll and Z-Gly-Pro-Leu-Gly-Pro as substrates. At least two major peaks express a proteolytic activity. The aminopeptidase activity able to hydrolyse LNA or Leu-Gly substrates seems in part associated with an iminopeptidase activity able to hydrolyse Pro-Leu. This was confirmed after isoelectrofocusing of the same extract since both activities were found in the same peak with an isoelectric point of 5.7. Two other amino-peptidase peaks were found at basic pH of which one strongly hydrolysed Leu-Gly whereas LNA was only weakly degraded.

Five caseolytic and four azocollytic activities were found. Although the relationships between the two activities are not clearly defined the major caseolytic

Table 2. Effect of some inhibitors on the main protease and peptidase activities of an adult worm extract after isoelectric focusing over the pH range 3–10. After hydrolysis in the presence of inhibitors the hydrolysate was characterized in BAW on Whatman No.3 paper for 10 hr and revealed by a 1% ninhydrin solution in acetone. The degree of hydrolysis was estimated and graded as, + hydrolysis comparable with the control without inhibitor, ± partial inhibition and − total inhibition

Isoelectrofocusing fractions	Substrates	Activity in the presence of inhibitors (10^{-3} M)		
		O-phenanthroline	PCMB	PMSF
10 (pHi 5.4)	Z-Gly-Pro-Leu-Gly-Pro	±	−	−
12 (pHi 5.7)	Leu-Gly	−	±	±
12 (pHi 5.7)	Pro-Leu	−	+	+
23 (pHi 7.6)	Leu-Gly	+	±	±

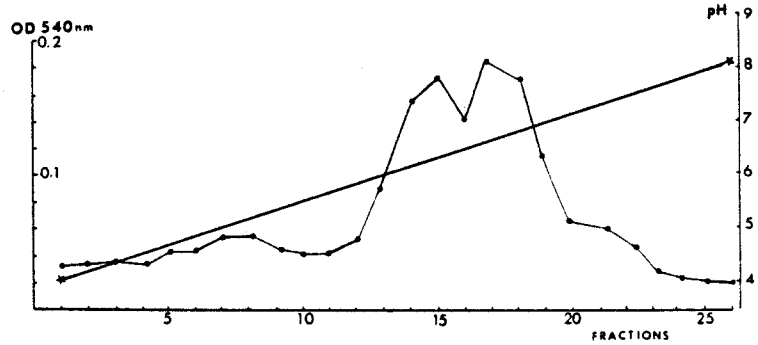

Fig. 14. Preparative isoelectric focusing of adult homogenate over the pH range 3–10. Fractions were assayed for azocoll activity by absorbance at 540 nm.

activity was situated at pH 4.4 while azocoll was mainly degraded by two activities found at pH 6.5 and 6.8. These results should be compared with those obtained with cercarial proteolytic enzymes (Stirewalt, 1978; Dresden & Asch, 1972; Landsperger *et al.*, in press) or with adult worm acidic proteases (Grant & Senft, 1971; Dresden & Deelder, 1979). The neutral pH activity of the enzymes studied during this work excludes in large part the acidic proteases previously described. In contrast, the maintenance of some cercarial neutral proteases during the mammalian development stages has to be considered.

The purification and the biologic relevance of these neutral enzymes is actually under investigation. The possible application of some of these enzymes as parasite antigens or their regulatory function in the immune response will be considered.

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DISCUSSION et CONCLUSION

Nous commencerons par discuter des résultats obtenus et des informations qu'ils apportent avant de conclure sur le modèle biologique plus général dans lequel ils s'inscrivent.

Ces travaux concernent donc essentiellement la régulation de la réponse immune de l'hôte mammifère par les protéases sécrétées par le parasite :

- soit directement en stimulant les éosinophiles ;
- soit indirectement en inhibant les macrophages par les peptides des IgG après leur clivage. Par ailleurs, ce clivage a également pour conséquence l'activation du complément par la voie classique sur l'intermédiaire du fragment Fc maintenu en place.

Ces travaux n'excluent pas d'ailleurs que les mêmes protéases puissent intervenir dans les deux cas. Les études biochimiques en cours devraient nous renseigner sur ce point.

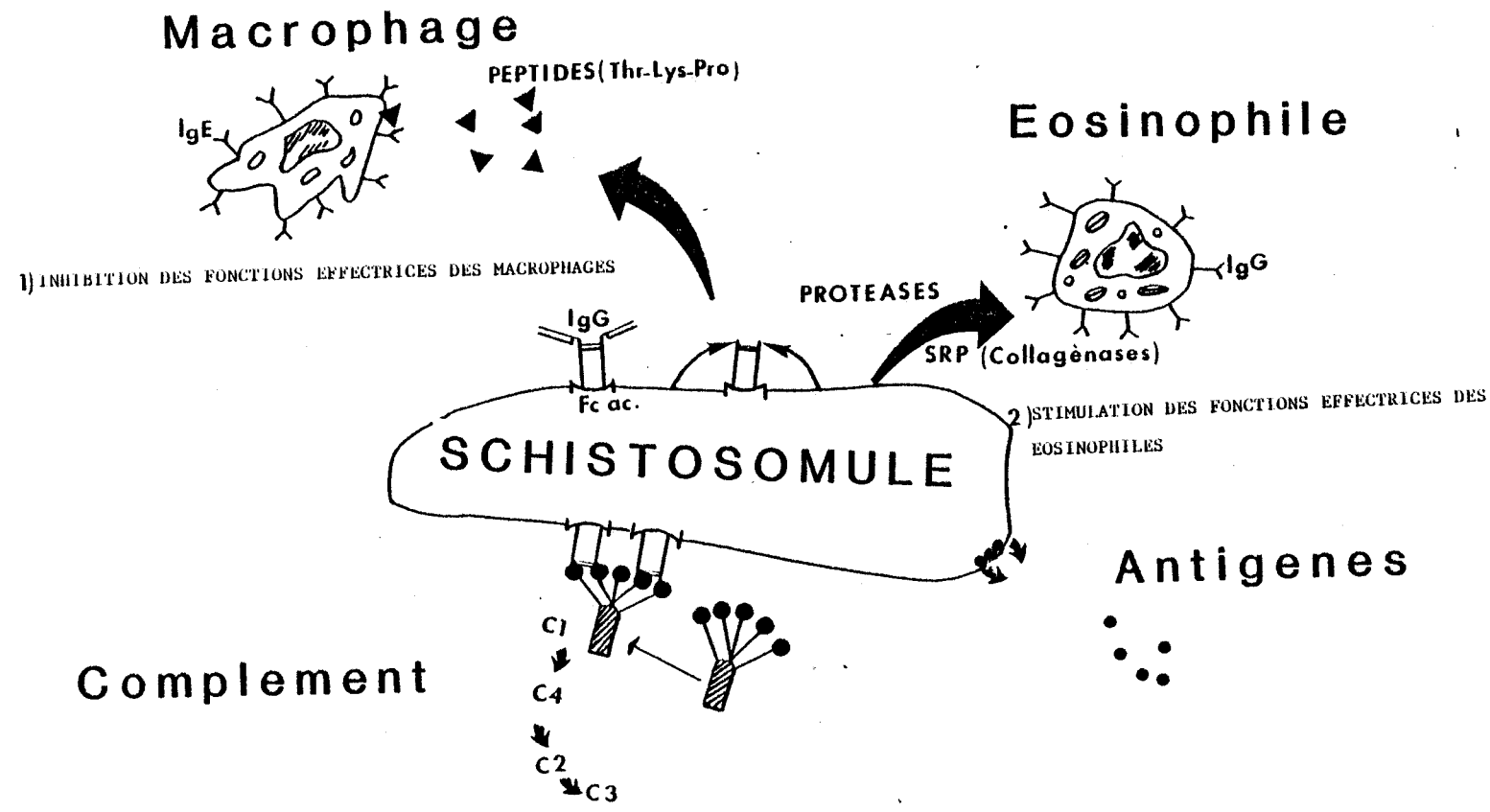
Dans le but de rendre cette discussion plus claire nous discuterons séparément ces deux mécanismes.

A) LE CLIVAGE DES IgG ET SES INCIDENCES SUR LA REPONSE IMMUNE

A partir de différentes techniques biologiques et biochimiques nous avons pu mettre en évidence que les IgG après leur fixation sur la membrane de la larve du schistosome sont clivées par des enzymes protéolytiques sécrétées par les schistosomules. Ce clivage a pour conséquence de libérer des peptides d'IgG dans le microenvironnement parasitaire tout en maintenant en place le fragment Fc de la molécule sur la membrane du parasite. L'intérêt de ce mécanisme nous est apparu lorsque nous avons pu mettre en

La chance favorise seulement les esprits préparés.

L. Pasteur



1) INHIBITION DES FONCTIONS EFFECTRICES DES MACROPHAGES

2) STIMULATION DES FONCTIONS EFFECTRICES DES EOSINOPHILES

3) ACTIVATION DU COMPLEMENT PAR LA VOIE CLASSIQUE

MODULATION DES FONCTIONS EFFECTRICES DES MACROPHAGES ET DES EOSINOPHILES PAR LES SCHISTOSOMULES. (recapitulatif)

évidence que les peptides ainsi libérés ont une activité fortement inhibitrice de l'activité effectrice des macrophages dépendant des IgE et plus généralement des mécanismes intervenant au cours de la phagocytose. Le parasite peut donc utiliser à son profit les produits de dégradation d'une molécule d'hôte comme les IgG, dont la concentration sérique est très importante, après leur fixation sur un accepteur de membrane spécifique, au moins vis-à-vis des autres isotypes (Torpier et coll., 1979). La régulation de certaines fonctions cellulaires par des peptides de diverses protéines a été déjà décrite dans de nombreux modèles. Dans le modèle schistosome par exemple comme nous l'avons déjà mentionné, Capron et Coll. (1981) ont mis en évidence le rôle du tétrapeptide mastocytaire qu'est l'ECF-A comme activateur de l'expression des récepteurs pour les immunoglobulines sur la membrane des éosinophiles. Un autre exemple est le SDIP (Spleen Derived Inhibitory peptide) qui est un peptide splénique qui a la propriété d'inhiber in vivo et in vitro la production d'anticorps (Millerioux et coll., 1981). Des peptides de collagène peuvent être chimioattractants pour des monocytes sanguins dans les foyers inflammatoires (Postlethwaite et coll., 1976). Les activités chimiotactiques ou immunorégulatrices de peptides naturels ou synthétiques ont fait l'objet de nombreuses études pharmacologiques ces dernières années. Il ne nous parait pas nécessaire d'énumérer tous les peptides présentant un intérêt pharmacologique mais citons pour exemple le muramyl-dipeptide (Chedid et coll., 1976) qui est un immunostimulant bien connu et la cyclosporine A caractéristique par ses activités immuno-suppressives (BOREL, 1976).

D'autres études ont montré que des peptides issus des IgG peuvent être impliqués dans des processus d'immunorégulation. Un hydrolysat de la région Fc des IgG1 humaines par des protéases sécrétées par les macrophages exercent une activité stimulatrice de la réponse anticorps anti-erythrocytes par les lymphocytes B murins (Morgan et Weigle, 1980). La molécule responsable est un peptide de 14 000 de poids moléculaire basé sur la séquence des résidus 335 - 357 de la molécule d'IgG (Morgan et coll., 1982) ce qui correspond à peu près aux 24 premiers acides aminés du domaine CH3. Le peptide n'agit pas directement sur les cellules B mais par l'activation de cellules "T helper". Le fragment Fcγ par lui-même peut exercer une activité modulatrice de certaines fonctions macrophagiques. Il est notamment capable de stimuler la production de prostaglandine E par des monocytes (Passwell et coll., 1980). Par contre, il inhibe chez ces mêmes cellules, la synthèse d'enzymes lysosomiales comme le lysozyme et la phosphatase acide ainsi que le second composant du complément (Passwel et coll., 1980). Ces auteurs ne précisent pas toutefois si le fragment Fcγ agit après sa dégradation par les enzymes des cellules cibles ou comme molécule entière. En effet un tétrapeptide, la Tuftsine, situé dans le second domaine constant des IgG1 humaines est un peptide résultant de l'hydrolyse des IgG par des protéases de neutrophiles appelée pour la circonstance des "leucokininases". Ce peptide exerce une activité stimulatrice des diverses fonctions des phagocytes et plus particulièrement des neutrophiles et des macrophages (Najjar et Nishioka, 1970 ; Tzehoval et coll., 1978 ; Najjar, 1981). Il est donc clair que des peptides résultant du clivage d'une molécule d'IgG peuvent jouer le rôle d'immunorégulateur et nos résultats confirment ce fait.

En effet, nous montrons que des peptides résultant du clivage d'immunoglobulines par des protéases parasitaires sécrétées par les larves, sont inhibiteurs de différentes activités macrophagiques. Par ailleurs, lorsque des IgG sont hydrolysées par d'autres protéases que des protéases parasitaires (trypsine, chymotrypsine, papaïne), nous n'avons pas retrouvé une telle activité inhibitrice à partir des peptides en résultant. C'est donc la spécificité particulière des protéases parasitaires qui est responsable de l'originalité du ou des peptides actifs. La présence d'un peptide inhibiteur situé dans la région CH_2 des IgG nous a été suggérée par un certain nombre de faits.

1) Par la structure même des molécules d'IgG, la région CH2 étant la plus exposée aux clivages enzymatiques. Cette propriété a d'ailleurs été utilisée dans le passé pour déterminer la structure, non seulement des IgG mais également par la suite de toutes les immunoglobulines.

2) Par les connaissances acquises sur les protéases sécrétées par les larves de schistosome et notamment des activités iminopeptidases et collagénases capables de cliver des chaines peptidiques avant ou après des résidus prolines, ce qui n'est pas banal. Or cette partie charnière de la molécule est assez riche en proline.

3) De la présence dans cette région de la séquence acceptrice du C1q qui est une macromolécule dont l'encombrement nécessite une structure d'accueil en fonction de sa taille et sa conformation. Cette région est donc disponible pour des ligands polypeptidiques, même de haut poids moléculaire, et les protéases peuvent répondent à cette description.

4) La présence dans cette même séquence d'un tétrapeptide réputé immunostimulateur comme la Tuftsine confirme la possibilité de fixation pour des enzymes protéolytiques mais indique en plus que par cette région, une molécule à fonction anticorps révèle dans sa structure deux autres fonctions que la simple liaison avec un antigène : la fixation spécifique du premier composant du complément et la génération de peptides immunostimulateurs. Toutes ces informations sont en fait supportées par le même gène codant pour la région constante des IgG. La fonction immunostimulatrice de la Tuftsine ne pourra s'exprimer qu'après dégradation de la molécule par des enzymes d'une spécificité particulière, codés par le génome des seuls neutrophiles.

5) Enfin, une suite d'expériences dont l'approche un peu grossière ne nous avait pas permis d'exploiter avec certitude les résultats, nous a guidé vers la région CH2. Lorsque l'on effectue une chromatographie d'affinité d'un hydrolysat d'IgG sur une colonne de sépharose couplé à du C1q, une partie de l'effet inhibiteur de cet hydrolysat sur l'incorporation de glucosamine par des macrophages péritonéaux de rats est en partie supprimée. Nous en avons conclu à l'époque que "certains" peptides inhibiteurs de l'activité des macrophages restaient fixés sur la colonne. Le manque de reproductibilité de ces résultats nous interdisait leur publication, toutefois ils ont contribué à fixer notre attention sur cette région "chaude" de la molécule qu'est le site de fixation du C1q.

6) Enfin, nos résultats montrent que cette région de liaison du C1q est exposée après le clivage de la molécule d'IgG. Ceci a pour conséquence l'activation du complément par la voie classique. Or, les travaux de Lukas et coll. (1981) montrent que la fixation

de la molécule de C1q se fait essentiellement sur le fragment 279 - 288 de la molécule d'IgG et que les quatre résidus suivants (289 - 292) correspondant à la séquence de la Tuftsine (Thr-Lys-Pro-Arg) ne représentent que 10 % de la fixation du C1q évalués en inhibition compétitive. Le double mécanisme, formation de peptides inhibiteurs et activation du complément, peut être expliqué par un clivage dans la partie carboxyterminale de la séquence acceptrice du C1q sur la molécule d'IgG. En prenant comme modèle l'IgG1 humaine, ceci conduirait à la libération de Thr-Lys-Pro inhibiteur des macrophages en maintenant en place la structure R-Val-Asp-Gly-Val-Gly-Val-His-Asn-Ala-Lys-COOH, suffisant pour fixer le C1q et permettre ainsi l'activation du complément par la voie classique. La nécessité de la fixation préalable des IgG sur un accepteur membranaire apparait donc clairement en stabilisant et en orientant la molécule.

Par ce mécanisme, le parasite peut donc utiliser à son profit des molécules de l'hôte et pas seulement pour masquer ses propres antigènes, mais dans un processus plus dynamique lui permettant d'intervenir sur les macrophages dont le rôle effecteur du moins in vitro, n'est plus contestable. C'est par la transformation des IgG par des protéases de spécificité "parasitaire" que la larve utilise une molécule d'hôte en faisant exprimer des peptides contenus dans sa séquence. Or ces peptides sont capables d'exercer une activité bien distincte de leur molécule d'origine qui ne pourrait en aucun cas l'exprimer sans l'hydrolyse conduisant à leur libération. Leur expression est la conséquence directe de l'action des protéases parasitaires, qui, par leur spécificité, fait exprimer une information potentielle contenue dans la structure d'une molécule protéique. Dans les mêmes conditions, la leukokininase des neutrophiles libère le tetrapeptide Tuftsine dont l'effet est complètement

inverse en ce sens que ce peptide est un activateur de l'activité des phagocytes. Ce processus est donc vraisemblablement un mécanisme naturel de régulation que le parasite détourne à son profit. Ce n'est pas sans rappeler l'infection virale dont l'intégration génomique favorise la prise en charge et le contrôle de l'expression génétique de l'hôte. La différence essentielle entre les deux modèles de parasitisme est que l'un intervient directement à la source de l'information biologique, donc au niveau des gènes de l'hôte, l'autre ne peut intervenir que sur leur produits. Toutefois, ces produits portent eux-même une information qui est réprimée tant que les conditions extérieures ne permettent pas leur libération sous forme de fragments peptidiques. Le parasite helminthe peut donc intervenir à un "troisième niveau génétique" où l'expression de tel ou tel peptide, d'IgG par exemple, dépendra de la spécificité des protéases environnantes Ceci naturellement permet des possibilités d'adaptation extragénomiques dont le parasite peut bénéficier grâce à la conservation de la spécificité des enzymes impliqués. Il n'est pas exclu que des peptides d'autres molécules d'hôte après leur clivage par les mêmes protéases ou d'autres, exercent également des activités immunorégulatrices et des travaux visant à leur mise en évidence sont actuellement en cours.

B - LE ROLE DES PROTEASES PARASITAIRES DANS L'ACTIVATION DES EOSINOPHILES

Nos résultats montrent donc que les facteurs thermosensibles sécrétés par le schistosome sont des enzymes protéolytiques de spécificité collagénase. La présence de telles protéases chez le

schistosome n'est pas très étonnante compte-tenu des diverses migrations que le parasite doit effectuer depuis sa pénétration transcutanée jusqu'au stade adulte. La traversée des différents tissus nécessite en effet la présence d'un matériel enzymatique adapté et les collagénases en font partie. Ce qui peut paraitre étonnant, c'est que ces collagénases exercent une activité de régulation du système immunologique et surtout un rôle stimulant sur l'une des cellules effectrices, principalement impliquée dans la réponse cellulaire anti-schistosome qu'est l'éosinophile.

Des protéases sont souvent mentionnées comme étant impliquées dans les mécanismes régulant les interactions cellulaires. Leur présence est mise en évidence par l'utilisation d'inhibiteurs ou en testant directement l'activité protéolytique des facteurs étudiés. Des lymphokines ont été décrites comme ayant des activités protéasiques. Par exemple, le LIF (Leukocyte inhibitory factor) est une sérine estérase (Goetzl et Rocklin, 1978), l'action stimulante du TRF sur les lymphocytes B (T cell release factor) est inhibée par des inhibiteurs protéasiques (Miki et coll., 1982). Il a également été montré qu'une monokine comme le LAF (Lymphokine Activating Factor) a des activités carboxypeptidase, et que la carboxypeptidase B peut être substituée à ce facteur et exercer la même activité stimulante de la prolifération des lymphocytes T (Dessaint et coll., 1979). L'utilisation des protéases comme immunorégulateur n'est donc pas une exclusivité du parasite. Dans la plupart des cas, l'activité immunorégulatrice est annulée par l'inhibiteur protéasique correspondant, ce qui signifie bien que leur activité biologique est associée à leur activité enzymatique. Nous avons, pour notre part, montré que le Trasylol peut inhiber l'effet stimulant exercé

par le SRP sur les fonctions effectrices des éosinophiles et que la spécificité collagénase elle-même est impliquée puisque l'on peut remplacer le SRP par une collagénase bactérienne purifiée et obtenir le même résultat. Dans la mesure où un tétrapeptide comme l'ECFA-A peut exercer une activité analogue sur les éosinophiles on est en droit de penser que probablement les protéases exercent leur activité stimulatrice par l'intermédiaire de leur activité enzymatique. On peut penser que la cible membranaire sera clivée et dégradée et la molécule active en elle-même pourrait être un peptide formé par l'hydrolyse du substrat cellulaire par la collagénase. On en revient donc à un mécanisme du même type que celui discuté dans le chapitre précédent. Ceci n'est qu'une hypothèse mais elle n'est pas sans rappeler les travaux récents effectués sur les récepteurs de l'insuline (Larner et coll., 1982). En effet, ces travaux mettent en évidence que le récepteur pour l'insuline est une sérine protéase membranaire dont le rôle consiste essentiellement à fixer et cliver la molécule d'insuline en fragments peptidiques, chacun porteur d'un message spécifique à la cellule. En présence de DFP, ce message ne pourra être transmis puisque la molécule d'insuline n'est pas clivée. On avait longtemps pensé que l'insuline elle-même était une protéase puisque son activité était inhibée par des inhibiteurs protéasiques. C'est son récepteur membranaire qui en était une. Cet exemple extrait d'un mécanisme non immunologique met bien en évidence :

1) L'effet indirect d'un facteur par l'intermédiaire de protéases de spécificité très stricte ;

2) Que l'information peut être apportée par des produits de clivage d'une protéase au niveau cellulaire.

Dans notre cas, il faudrait imaginer que le mécanisme est inversé et que ce soit le substrat qui soit fixé et la protéase circulante. Ceci n'exclut en rien que l'on puisse aboutir au même résultat.

Ce mécanisme conduisant à la stimulation d'une cellule immunocompétente, montre à quel point le parasite peut intervenir sur la réponse immune de son hôte et est à rapprocher de la notion d'immunité concomitante décrite dans le chapitre d'introduction. En effet, des produits sécrétés par des vers adultes peuvent réguler des mécanismes effecteurs dirigés contre les larves du parasite. Dans le cas de l'immunité concomitante, on parlera d'antigènes exprimés avec toute la notion de spécificité antigénique, dans notre cas de facteurs sécrétés,stimulants spécifiques des seuls éosinophiles. Il convient toutefois de ne pas discuter ce mécanisme au niveau d'un individu mais de l'espèce Schistosoma mansoni. Le maintien du parasitisme dépendra de la survie de l'hôte, c'est évident. Par ailleurs la survie de l'espèce dépendra de sa faculté à se reproduire et à assurer sa survie. Or, ce sont les adultes qui perennisent l'espèce. Un accroissement de la charge parasitaire de l'hôte par des infestations nouvelles risque à court terme de déséquilibrer l'écosystème, ce qui de toute façon aboutira à une "stérilisation" des reproducteurs ou à leur mort. Dans les régions endémiques les risques d'infections multiples sont grands et il n'est pas étonnant que la pression sélective ait maintenu des mécanismes visant à stimuler les fonctions immunitaires susceptibles de tuer les larves infestantes. Les adultes eux-même protégés de la réponse immune susciteront des mécanismes de défense dirigés contre des antigènes que les schistosomules exprimeront sur leur membrane. Ils pourraient donc de plus, excréter des "adjuvants" capables de stimuler

les fonctions effectrices des éosinophiles. Les schistosomules eux-mêmes sécrètent les protéases stimulantes, qui ne seront efficaces que dans le cas d'une réinfection puisque les éosinophiles ne sont cytotoxiques qu'en présence d'anticorps anaphylactiques spécifiques de classes G ou E, et la réponse anticorps apparait assez tardivement après la première infection.

C) CONCLUSION GENERALE

Ces travaux mettent donc en évidence que le schistosome peut moduler la réponse immunitaire soit en inhibant certaines cellules soit en en activant d'autres. L'interprétation réelle de ces mécanismes est encore hypothétique et la nature exacte des relations hôte-parasite est très loin d'être comprise compte-tenu de leur grande complexité. Il faudra néanmoins admettre que si la pression sélective a maintenu des mécanismes visant à réguler la réponse immune, c'est qu'ils ont leur raison d'être, même si nous n'avons pas encore les moyens de les interpréter dans un contexte général. De nombreux paramètres doivent intervenir comme la charge parasitaire initiale, l'état général de l'hôte au moment de la première infection, les taux d'hormones stéroïdes circulants, l'âge, etc... et chacun devra être étudié séparément. Nos travaux n'ont comme ambition

que de présenter des observations originales qui ne représentent qu'une infime partie de la réalité biologique. Il est probable toutefois que le modèle schistosome puisse apporter des connaissances sur les mécanismes de régulation de la réponse immunitaire insoupçonnées par l'utilisation de modèles traditionnels. Les études pharmacologiques engagées dans notre laboratoire montrent déjà en tout cas que si on considère un schistosome comme on a considéré les plantes médicinales, il révèle un potentiel immunopharmacologique important. Il serait bien dommage de ne pas exploiter ces facteurs régulateurs que l'espèce a maintenu avec succès depuis des milliers de générations et qui ont malheureusement fait leurs preuves au détriment d'un grand nombre de malades.

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