INTERACTIONS BETWEEN HUMAN EOSINOPHILS AND SCHISTOSOMULA OF *SCHISTOSOMA MANSONI*

II. The Mechanism of Irreversible Eosinophil Adherence*

By ANTHONY E. BUTTERWORTH,‡ MATHEW A. VADAS, DONALD L. WASSOM, ALAIN DESSEIN, MAUREEN HOGAN, BARBARA SHERRY, GERALD J. GLEICH, AND JOHN R. DAVID

From the Department of Medicine, Harvard Medical School, and the Robert B. Brigham Hospital, a Division of the Affiliated Hospitals Center Inc., Boston, Massachusetts; and the Department of Medicine and Immunology, Mayo Foundation, Rochester, Minnesota

We have reported elsewhere (1)[1] that normal human eosinophils adhere to schistosomula of *Schistosoma mansoni*, in the presence of heat-inactivated sera from patients with schistosomiasis, to a much greater extent than do neutrophils. This finding was surprising, because neutrophils are generally considered to bear more Fc receptors (FcR) for bound IgG than do eosinophils (2–5). On further study, it became apparent that the initiation of adherence by both cell types was a temperature-independent reaction involving the cells' FcR, and that the eosinophil, but not the neutrophil, then underwent a further, temperature-dependent step which rendered its binding progressive and irreversible.[1]

We considered that this preferential binding of eosinophils to antibody-coated schistosomula might, in part, account for the selective damaging effect of the eosinophil, in comparison with the neutrophil (1), and that it was therefore important to study the mechanism of the irreversible step in more detail. Earlier work by ourselves (6–8) and others (9, 10) had shown that damage induced by eosinophils was associated with cell attachment, followed by degranulation and the release of granule contents onto the surface of the organism. This damage was attributable, at least in part, to the release of one major component of the eosinophil granule, the major basic protein (MBP)[2] (11, 12), onto the surface of the organism (13). In contrast, adherent neutrophils showed no signs of degranulation detectable at the electron microscopical level: instead, these cells formed fusions with the outer bilayer of the schistosomulum

* Supported by grants from the Wellcome Trust, the Edna McConnell Clark Foundation, the Rockefeller Foundation, and the National Institute of Allergy and Infectious Diseases.

‡ Address for reprint requests: Department of Pathology, Tennis Court Road, Cambridge CB2 1QP, England.

[1] Vadas, M. A., A. E. Butterworth, B. Sherry, A. Dessein, M. Hogan, D. Bout, and J. R. David. Interactions between human eosinophils and schistosomula of *Schistosoma mansoni*. I. Stable and irreversible antibody-dependent adherence of eosinophils to schistosomula. *J. Immunol.* In press.

[2] *Abbreviations used in this paper:* Con A, concanavalin A; E/LAC, Earle's balanced salt solution with 0.5% lactalbumin hydrolysate; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; MBP, eosinophil major basic protein; MEM, Eagle's minimal essential medium; NEM, *n*-ethylmaleimide; PBS, phosphate-buffered saline, pH 7.4.

membrane, resulting in the elimination of a membrane layer (14).[3]

It seemed reasonable to postulate that the irreversible nature of eosinophil adherence was also associated with degranulation, and that the released granule contents, in particular MBP, might serve as a ligand, binding together the two negatively charged surfaces. This material would both prolong the adherence of cells that are already attached and enhance the adherence of new cells coming into the site of the reaction, thereby promoting a cascade effect.

In this paper, we describe attempts to test this hypothesis, in particular by the use of concanavalin A (Con A) instead of antibody as a ligand to mediate eosinophil or neutrophil adherence. We have found that eosinophils bound via Con A fail to degranulate, and that this is associated with a lack of damage to the parasite and a complete reversibility of adherence with an appropriate competing agent. However, if degranulation of Con A-bound eosinophils is induced with the calcium ionophore A23187, such cells now damage the schistosomulum, and their adherence is no longer reversible by a competing agent. These findings support our contention that both eosinophil-mediated damage and the irreversible nature of eosinophil adherence are dependent on degranulation.

Materials and Methods

Media and Reagents. The following media were used in these experiments: Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.), buffered with 25 mM Hepes and containing penicillin, 100 u/ml, and streptomycin, 100 μg/ml; MEM/fetal calf serum (FCS), MEM supplemented with 10% FCS (Flow Laboratories, Inc., Rockville, Md.) heat-inactivated at 56°C for 1 h; Earle's balanced salt solution with 0.5% lactalbumin hydrolysate (E/LAC) (Grand Island Biological Co.), supplemented with 10% heat-inactivated FCS and antibiotics as above; phosphate-buffered saline (PBS), pH 7.4; and Hanks' balanced salt solution (HBSS).

Con A (type IV: Sigma Chemical Co., St. Louis, Mo.) was prepared as a stock solution of 2 mg/ml in PBS and was stored in aliquots at −20°C. Appropriate dilutions were made in MEM/FCS immediately before use. A23187 was kindly donated by Lilly Research laboratories to Dr. E. Martz, and was dissolved in dimethylsulphoxide at 10 mg/ml. Aliquots were stored in light-proof vessels, and were diluted in MEM/FCS immediately before use. Alpha-methyl-mannoside (Sigma Chemical Co.) was freshly prepared in MEM/FCS to yield a final concentration of 10^{-1} M. Protamine (free base, Sigma Chemical Co.) and *n*-ethylmaleimide (Sigma Chemical Co.) were freshly dissolved and diluted in MEM to yield the final concentration shown in the experiments.

Guinea pig eosinophil MBP was prepared as described in detail by Gleich et al. (11, 12, 15, 16), and was stabilized by alkylation of the two free sulphydryl groups (13). The alkylated material was dialysed against 0.1 M NH_4HCO_3, and lyophilized. Immediately before use, the MBP was dissolved in MEM.

Schistosoma mansoni Life Cycle and Preparation of Schistosomula. A Puerto Rican strain of *S. mansoni* is routinely maintained in this laboratory, and schistosomula were prepared by allowing infective cercariae to penetrate rat skin in vitro (17, 18). Such schistosomula were stored overnight at 4°C in E/LAC/FCS, and were washed in MEM/FCS immediately before use (1).

Human Sera. Sera from patients with *S. mansoni* infection were used as a source of antibody. These anti-schistosomular sera were heat-inactivated at 56°C, and showed no direct toxicity to

[3] Caulfield, J. C., G. Korman, A. E. Butterworth, M. Hogan, and J. R. David. The adherence of neutrophils, eosinophils and erythrocytes to schistosomula: evidence for membrane fusion between cells and a parasite. Submitted for publication.

schistosomula. Dilutions were chosen which gave high levels of eosinophil-mediated adherence and damage (1).

Preparation of Human Leukocytes. Eosinophils and neutrophils were purified from normal human peripheral blood by centrifugation of leukocyte-rich suspensions over discontinuous metrizamide gradients, as described elsewhere (1). Preparations containing >90% eosinophils or neutrophils were routinely obtained, and differentials are given in the figures and tables for each preparation tested. Cells recovered from the gradients were washed twice in MEM/FCS and resuspended at concentrations ranging in different experiments from 1 to 8×10^6 cells/ml.

Microscopical Assay for Cell Adherence to Schistosomula. Cell adherence to schistosomula was determined after incubation at 37°C of various combinations of schistosomula, cells, antiserum, or Con A, and additional reagents (1). Aliquots of 50 μl of each component of the reaction were mixed in 7 × 38-mm plastic tubes. After varying periods of time, usually 2 h, the contents of each tube were transferred to a slide previously coated with 0.1% toluidine blue in methyl alcohol, and each organism in each of two or three replicate preparations was examined for cell adherence at a magnification of ×100. For each test condition in each experiment, cumulative plots were prepared of the percentage of organisms bearing 3 or more, 5 or more, 10 or more, or 20 or more tightly adherent cells (example given in Fig. 2). In some experiments, it was necessary to choose one or two criteria for clarity of presentation of the data, but the results were consistent whichever criterion was chosen.

Microscopical Assay of Cell-mediated Damage to Schistosomula. Preparations containing schistosomula, cells, and other reagents as above were incubated for 1 or 2 d at 37°C in humidified plastic boxes. At the end of the incubation period, the contents of each tube were resuspended and transferred to slides coated with toluidine blue, as described above. Organisms were scored as damaged if they were immotile, misshapen, and had taken up toluidine blue in an intense and granular fashion (1).

Radioimmunoassay for Released MBP. The release of MBP into the supernates of cultures containing eosinophils, as a marker for eosinophil degranulation, was detected by a radioimmunoassay described in detail elsewhere (13, 16). The amount of MBP released into each supernate was calculated by interpolation in a standard curve, and is expressed as nanograms MBP per milliliter supernate.

Analysis of Results. For experiments involving cell adherence or cell-mediated damage, mean values of two or three replicate tubes were compared by Student's *t* test.

Results

Adherence of Eosinophils and Neutrophils to Intact or Modified Schistosomula. The differences previously observed in the antibody-dependent adherence of eosinophils and neutrophils (1)[1] reflected the ability of these cells to interact with living schistosomula. When preparations of schistosomula contained an unusually high proportion of spontaneously dead organisms, the antibody-dependent adherence of eosinophils to live and dead organisms respectively was comparable (Table I), although there was a greater degree of antibody-independent adherence to dead organisms. For neutrophils, both the antibody-independent and, more strikingly, the antibody-dependent adherence was markedly greater to dead than to live schistosomula. Comparable observations were made when the surfaces of schistosomula were artificially modified by *n*-ethylmaleimide (NEM). Pretreatment of schistosomula with varying concentrations of NEM was associated with a marked increase in the antibody-dependent adherence of neutrophils in a dose-dependent fashion (64 ± 12%, 29 ± 12%, and 11 ± 1% adherence to organisms treated with 10^{-3}, 10^{-4}, and 10^{-5} M NEM, respectively, in contrast to 2 ± 2% adherence to control organisms). Eosinophils showed a slight but less marked increase in antibody-dependent adherence to NEM-treated organisms (40 ± 14%, 47 ± 19%, 36 ± 16%, and 13 ± 13% adherence to schistosomula treated with 10^{-3}, 10^{-4}, or 10^{-5} M NEM, respectively, or with control buffer).

TABLE I

Adherence of Eosinophils and Neutrophils to Living or to Spontaneously Dead Schistosomula

Cells*	Antiserum‡	Schistosomula§	Percentage of schistosomula bearing adherent cells‖ 5+	10+	20+	Percentage of dead schistosomula§
Eosinophils	+	Live	88 ± 5¶	76 ± 5	51 ± 14	24 ± 4
		Dead	100 ± 0	94 ± 6	69 ± 30	
	−	Live	2 ± 2	0	0	28 ± 3
		Dead	54 ± 25	23 ± 13	3 ± 3	
Neutrophils	+	Live	40 ± 10	13 ± 3	3 ± 1	22 ± 4
		Dead	97 ± 4	97 ± 4	97 ± 4	
	−	Live	0	0	0	23 ± 8
		Dead	78 ± 10	47 ± 26	14 ± 12	

* Eosinophils (96% eosinophils, 4% neutrophils) or neutrophils (92% neutrophils, 3% eosinophils, 5% mononuclears) were tested at an effector to target ratio of 1,000:1.

‡ Heat-inactivated human antischistosomular serum (+) or medium as control (−).

§ A preparation of schistosomula which contained ~25% dead organisms at the time of testing (preparations normally contain 5–10% dead organisms).

‖ Schistosomula were incubated with or without antiserum and with either eosinophils or neutrophils for 2 h at 37°C. The percentages of living and of dead schistosomula bearing 5 or more, 10 or more, or 20 or more adherent cells were then scored.

¶ Mean ± SD for three replicate tubes.

Subsequent experiments were designed to test the hypothesis that the progressive and irreversible adherence to living schistosomula of eosinophils, in contrast to neutrophils, was associated with degranulation. First, attempts to mimic the effect of eosinophil degranulation by incubation of schistosomula with polycations led to an enhanced adherence of eosinophils and, to a much greater extent, of neutrophils. Second, the adherence of eosinophils via Con A was shown to be independent of degranulation and fully reversible, but induction of degranulation in Con A-bound eosinophils converted the reaction into one which was no longer reversible.

Enhancement of Antibody-dependent Adherence of Eosinophils and Neutrophils by Polycations. Addition of subtoxic concentrations of protamine to schistosomula led to an increased antibody-dependent adherence both of neutrophils and of eosinophils, with a smaller effect on antibody-independent adherence (Table II). A comparable, although less marked, increase in adherence of unpurified leukocytes and of purified eosinophils and neutrophils was observed in the presence of guinea pig MBP.

Con A-mediated Adherence to Schistosomula of Eosinophils and Neutrophils. Addition of Con A to schistosomula induced, in a dose-dependent fashion, a marked and comparable adherence of both eosinophils and neutrophils (Fig. 1). Similar results were obtained in two further titrations. When schistosomula were preincubated with Con A for 1 h and then washed, the subsequent adherence of eosinophils and of neutrophils was reduced by comparison with the unwashed preparations, suggesting that the Con A eluted rapidly from the surface of the organism (data not shown). In subsequent experiments, therefore, the Con A was allowed to remain present throughout the reaction.

Although the Con A-induced adherence of both neutrophils and eosinophils was as good as or better than that seen in the presence of anti-schistosomular serum, both at

TABLE II

Enhancement of Eosinophil and Neutrophil Adherence by Polycations

Exp.	Cells‡	Reagent	μg/ml	Percent adherence*			
				Antiserum* +		−	
				5+*	10+*	5+	10+
1	Eosinophils	Protamine	0	42 ± 8§	20 ± 5	1 ± 1	0
			5	38 ± 3	15 ± 11	6 ± 0	1 ± 1
			50	*86 ± 8*‖	*58 ± 8*‖	15 ± 8	2 ± 2
			500¶	51 ± 11	11 ± 7	1 ± 1	20 ± 2
	Neutrophils	Protamine	0	6 ± 0	1 ± 1	0	0
			5	*34 ± 6*†	13 ± 7	0	0
			50	*56 ± 2*‖	32 ± 4	*8 ± 3*‖	0
			500¶	*73 ± 8*‖	52 ± 1	*22 ± 3*‖	9 ± 12
2	Eosinophils	Protamine	0	15 ± 3	8 ± 2	4 ± 5	0
			5	23 ± 4	15 ± 4	0	0
			50	*60 ± 19*‖	*27 ± 2*‖	5 ± 3	0
	Neutrophils	Protamine	0	30 ± 3	20 ± 1	2 ± 3	2 ± 2
			5	*53 ± 6*‖	29 ± 5	5 ± 1	0
			50	*67 ± 1*‖	*44 ± 1*‖	6 ± 7	4 ± 6
3	Mixed	MBP	0	10 ± 2	NM**	2 ± 2	NM
			10	*31 ± 3*‖	NM	3 ± 1	NM
4	Mixed	MBP	0	55 ± 7	42 ± 13	11 ± 5	11 ± 5
			25	*72 ± 5*‖	57 ± 7	*28 ± 5*‖	19 ± 3
5	Eosinophils	MBP	0	89 ± 5	51 ± 7	0	0
			16	98 ± 2	*82 ± 1*‖	0	0
	Neutrophils	MBP	0	15 ± 9	3 ± 2	0	0
			16	31 ± 5	*11 ± 2*‖	0	0

* Schistosomula were incubated in the presence (+) or absence (−) of human anti-schistosomular serum, and with either mixed leukocytes or eosinophil-rich or neutrophil-rich cell preparations at a ratio of 1,000:1. In each case, the schistosomula were preincubated for 5 min with varying concentrations of protamine or of eosinophil MBP before addition of the cells. Adherence was scored after 2 h of incubation as the percentage of organisms bearing 5 or more (5+) or 10 or more (10+) adherent cells.

‡ Eosinophils: exp. 1, 98% eosinophils, 2% neutrophils; exp. 2, 94% eosinophils, 6% neutrophils; exp. 5, 94% eosinophils, 6% neutrophils. Neutrophils: exp. 1, 96% neutrophils, 3% eosinophils, 1% mononuclears; exp. 2, 97% neutrophils, 3% mononuclears; exp. 5, 98% neutrophils, 1% eosinophils, 1% mononuclears. Mixed: exp. 3, 5% eosinophils, 61% neutrophils, 34% mononuclears: exp. 4, 7% eosinophils, 72% neutrophils, 21% mononuclears.

‖ Values in italics differ from the appropriate medium only ("0") control at $P < 0.05$ or less.

¶ In preparations containing 500 μg/ml protamine, the schistosomula were motile but markedly damaged.

** NM, not measured.

2 h of incubation and also after 24 or 48 h of incubation, there was no evidence of damage to schistosomula with either cell type after 24 or 48 h of incubation (Table III). In the same experiments, it was observed that the eosinophils, but not the neutrophils, exerted a marked effect in the presence of anti-schistosomular serum. In three of these experiments (1, 2, and 4) it was observed that eosinophils bound via Con A failed to release MBP into the culture supernate, showing that they had failed to degranulate. These results are described in greater detail below (Table IV).

Reversibility of Con A-mediated Eosinophil and Neutrophil Adherence by Alpha-methylmannoside. Because eosinophils which had been bound to schistosomula via Con A neither

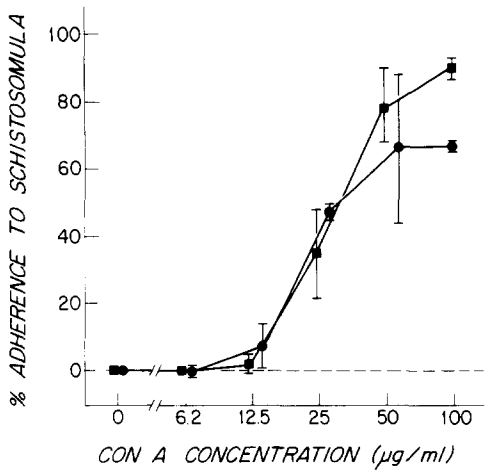

FIG. 1. Adherence of eosinophils and neutrophils to schistosomula in the presence of varying concentrations of Con A. Schistosomula were incubated with Con A and with cell preparations containing 95% eosinophils and 5% neutrophils (■) or 99% neutrophils and 1% eosinophils (●) at a ratio of 2,000:1. Adherence was scored after 2 h of incubation as the percentage of organisms bearing 10 or more adherent cells. Identical concentrations of Con A were used for eosinophils and neutrophils, respectively: values are slightly displaced to reveal the mean ± 1 SD for three replicate tubes.

degranulated, as indicated by a lack of MBP release, nor damaged the target schistosomula, it was predicted that their adherence to such schistosomula should be fully reversible by an appropriate competing agent. To test this prediction, schistosomula were incubated with Con A and with either eosinophils or neutrophils for 1 h, and the level of adherence was scored (Fig. 2). Alpha-methylmannoside (10^{-1} M), or medium as control, was then added to separate groups of tubes, which were incubated for a further 1 h before adherence was scored. Both for neutrophils and for eosinophils, the adherence that was observed after 1 h of incubation was reduced to background levels by addition of alpha-methylmannoside and incubation for a further 1 h. This finding for eosinophils is in marked contrast to the results for antibody-dependent adherence described elsewhere,[1] in which eosinophil adherence was not reversible after 1 h of incubation by addition of *Staphylococcus aureus* protein A, whereas neutrophil adherence was fully reversible.

A similar, complete reversal of eosinophil adherence by alpha-methylmannoside was observed in three further experiments carried out under identical conditions (data not shown). It was also found that, as would be expected, the initiation of Con A-mediated adherence by both eosinophils and neutrophils was completely inhibited (0% adherence in two experiments) by addition of 10^{-1} M alpha-methylmannoside at the beginning of the incubation period. In addition, it was noted that alpha-methylmannoside failed to reverse the antibody-dependent adherence of neutrophils. In an experiment in which Con A-dependent adherence was reduced from 81 ± 15% after 1 h of incubation to 9 ± 1% adherence after incubation with 10^{-1} M alpha-methylmannoside for a further hour, antibody-dependent adherence under the same conditions rose from 49 ± 18% after 1 h to 80 ± 1% after incubation with alpha-methylmannoside for a further hour. This finding indicated that the effect of alpha-

TABLE III

Failure of Eosinophils and Neutrophils to Induce Damage when Bound to Schistosomula Via Con A

Exp.	Ligand*	Ratio*	Percent dead schistosomula*‡		
			Eosinophils §	Neutrophils‖	Medium
1	Con A	2,500:1	15 ± 1	16 ± 4	16 ± 1
	medium		18 ± 4	10 ± 5	11 ± 2
2	Con A		12 ± 1	14 ± 5	7 ± 1
	medium	1,000:1	9 ± 9	15 ± 2	13 ± 1
	antiserum		*27 ± 2*¶	14 ± 1	11 ± 6
3	Con A		6 ± 2	7 ± 4	3 ± 3
	medium	2,000:1	4 ± 2	3 ± 1	7 ± 1
	antiserum		*78 ± 6*¶	8 ± 4	6 ± 3
4	Con A		13 ± 7	7 ± 1	7 ± 3
	medium	4,000:1	14 ± 5	5 ± 4	7 ± 1
	antiserum		*95 ± 1*¶	11 ± 3	6 ± 2
5	Con A		14 ± 4	NT**	15 ± 3
	medium	2,500:1	12 ± 4	NT	13 ± 1
	antiserum		*27 ± 5*¶	NT	11 ± 4
	Con A + antiserum		*37 ± 1*¶	NT	10 ± 2

* Schistosomula were incubated with Con A (40 μg/ml), anti-schistosomular serum (antiserum) or medium and with either eosinophil-rich or neutrophil-rich cell preparations at varying effector to target ratios. Damage was scored after 1 d (exp. 2) or 2 d (exps. 1, 3, 4, 5) of incubation, and are shown as mean ± 1 SD for two (exps. 2, 3, 5) or three (exps. 1, 4) replicate tubes.

‡ In preparations containing Con A, it was observed that there were high levels of adherence of both neutrophils and eosinophils after 24 or 48 h of incubation, even though the organisms were not detectably damaged.

§ Exp. 1, 94% eosinophils, 6% neutrophils: exp. 2, 94% eosinophils, 5% neutrophils, 1% mononuclears: exp. 3, 96% eosinophils, 4% neutrophils: exp. 4, 92% eosinophils, 8% neutrophils: exp. 5, 96% eosinophils, 4% neutrophils.

‖ Exp. 1, 67% neutrophils, 12% eosinophils, 21% mononuclears: exp. 2, 99% neutrophils, 1% eosinophils: exp. 3, 83% neutrophils, 12% eosinophils, 5% mononuclears: exp. 4, 99% neutrophils, 1% eosinophils: exp. 5, 97% neutrophils, 3% mononuclears.

¶ Values in italics differ from the medium (no cells) controls at $P < 0.05$ or less.

** NT, not tested.

methylmannoside on Con A-dependent adherence was not simply attributable to a toxic effect of the hypertonic sugar solution on the cells, which rendered them unable to adhere.

Effects of A23187 on Con A-mediated Adherence of Eosinophils and Neutrophils. A further prediction from the hypothesis outlined above would be that, if eosinophils bound to schistosomula via Con A could be induced to degranulate, they should now no longer show a reversal of adherence in the presence of alpha-methylmannoside, and they should instead go on to damage the target schistosomula.

The most satisfactory of several methods tested to induced degranulation, as indicated by the release of MBP into the supernate, was the use of the calcium ionophore A23187. Two types of experiments were carried out to test the effect of this agent on the reversibility of adherence induced by Con A. In the first (Fig. 3), schistosomula were incubated in the presence or absence of Con A for 1 h, together with either eosinophil-rich or neutrophil-rich cell preparations. A point that is not

TABLE IV

A23187 Induces Eosinophil Degranulation and Converts the Con A-mediated Adherence of Eosinophils into a Lethal Reaction

Reagents	Percent dead schistosomula*			MBP release from eosinophils
	Eosinophils	Neutrophils	Medium	
				ng/ml
Con A	13 ± 7	7 ± 1	7 ± 3	16.7 ± 0.1
Con A + A23187 0.04 μg/ml	*34 ± 3*‡	9 ± 3	8 ± 3	*25.4 ± 0.3*§
Con A + A23187 0.1 μg/ml	*54 ± 7*‡	*14 ± 2*‡	9 ± 2	*47.1 ± 8.6*§
Medium	14 ± 5	5 ± 4	7 ± 1	23.6 ± 0.6
Medium + A23187 0.04 μg/ml	11 ± 7	9 ± 3	8 ± 1	20.0 ± 0.6
Medium + A23187 0.1 μg/ml	*33 ± 10*‡	9 ± 6	9 ± 2	*38.0 ± 1.1*§
Antiserum	*95 ± 1*‡	11 ± 2	6 ± 2	*30.4 ± 1.1*§

* Schistosomula were incubated with Con A (40 μg/ml), anti-schistosomular serum (antiserum) or medium, and with either eosinophil-rich (92% eosinophils, 8% neutrophils) or neutrophil-rich (99% neutrophils, 1% eosinophils) cell preparations at a ratio of 4,000:1, or with medium as control. After incubation for 1 h to permit Con A-mediated adherence, A23187 was added to some groups of tubes. After incubation for a further 18 h, an aliquot of the supernate of each tube was withdrawn for estimation of MBP release. Samples from each of three tubes were pooled, and the values shown represent mean ± 1 SD for duplicate measurements. Values in the neutrophil and medium groups were less than 2 ng/ml, except that the antiserum control groups gave a value of 6 ng/ml (see reference 14). Schistosomula were then incubated for a further 24 h: damage was scored microscopically, and is recorded as mean ± 1 SD for three replicate tubes.

‡ Values in italics differ from the appropriate no ionophore or no cells controls at $P < 0.05$ or less.

§ Values in italics differ from the appropriate Con A or Medium controls at $P < 0.05$ or less.

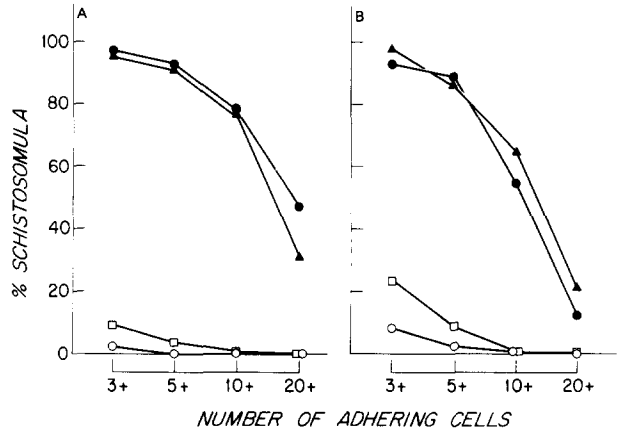

FIG. 2. Reversal of Con A-mediated adherence of eosinophil (A) and neutrophils (B) by alpha-methylmannoside. Schistosomula were incubated under different test conditions with eosinophils (95% eosinophils, 5% neutrophils) or with neutrophils (99% neutrophils, 1% eosinophils) at a ratio of 2,000:1. Adherence was scored in three replicate tubes in each group as the percentage of organisms bearing 3 or more (3+), 5 or more (5+), 10 or more (10+), or 20 or more (20+) adherent cells. ●, incubated with Con A, 40 μg/ml; adherence scored at 1 h. ▲, incubated with Con A, 40 μg/ml; adherence scored at 2 h. ○, incubated with medium; adherence scored at 2 h. □, incubated with Con A, 40 μg/ml, for 1 h, then with alpha-methyl-mannoside (10^{-1} M) for a further 1 h; adherence scored at the end of the 2nd h.

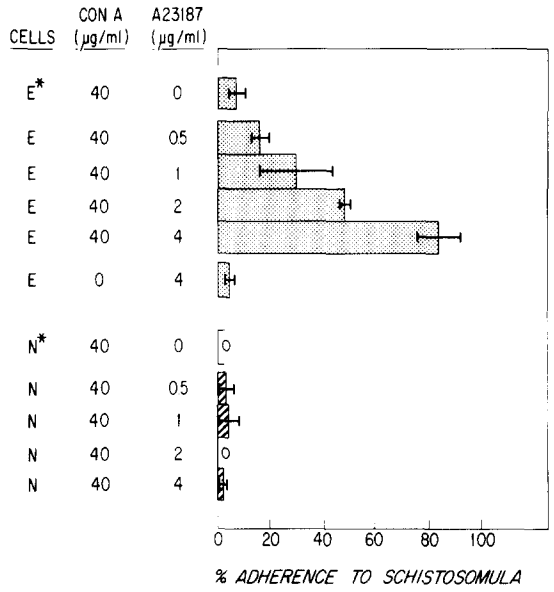

FIG. 3. Induction of degranulation with A23187 inhibits the spontaneous reversal of Con A-mediated eosinophil adherence that occurs after washing and incubation. Schistosomula were incubated with Con A (40 μg/ml) or with medium, and with either eosinophils (91% eosinophils, 9% neutrophils) or neutrophils (99% neutrophils, 1% eosinophils) at an effector to target ratio of 2,000:1 for 1 h at 37°C. A23187 was then added at the concentrations shown, and the preparations were incubated for a further ½ h. Schistosomula were then washed by four sedimentations at 1 *g* to remove the unbound cells, the excess Con A, and the ionophore. Preparations were then incubated for a further 18 h, and adherence was scored as the percentage of organisms bearing five or more adherent cells (mean ± 1 SD for two replicate tubes). At the end of the 1st h of incubation, before addition of the ionophore, individual aliquots were examined for adherence. Marked adherence was observed both with eosinophils and with neutrophils.

shown in Fig. 3 is that this procedure, as usual, induced a high level of adherence of both cell types. A23187 was then added to different groups of tubes in varying concentrations, and the preparations were incubated for a further ½ h. The schistosomula were then washed by four cycles of sedimentation for 3 min at 1 *g*, a procedure which served to remove the ionophore, the excess Con A, and the unbound cells. All tubes were then incubated for a further 18 h, before scoring for adherence. The neutrophils, as usual, showed a reversal of adherence, irrespective of whether A23187 had or had not been present. In contrast, A23187 prevented, in a dose-dependent fashion, the spontaneous reversal of eosinophil adherence that occurred after overnight incubation in control preparations which had not been treated with the ionophore.

In the second type of experiment, the ability of A23187 to prevent the acute reversal of adherence induced by alpha-methylmannoside was tested (Fig. 4). Schistosomula were incubated in the presence of Con A with either eosinophil-rich or neutrophil-rich cell preparations for 1 h. A23187, or medium as control, was then added, and the contents of each tube were gently resuspended and allowed to incubate for a further 1 h. Alpha-methylmannoside, or medium as control, was then added, and the contents of each tube were again resuspended and incubated for a further 1 h. Adherence was then scored in all preparations. In the case of neutrophils, the adherence induced by Con A was partially reversed simply by the presence of A23187, and there was no

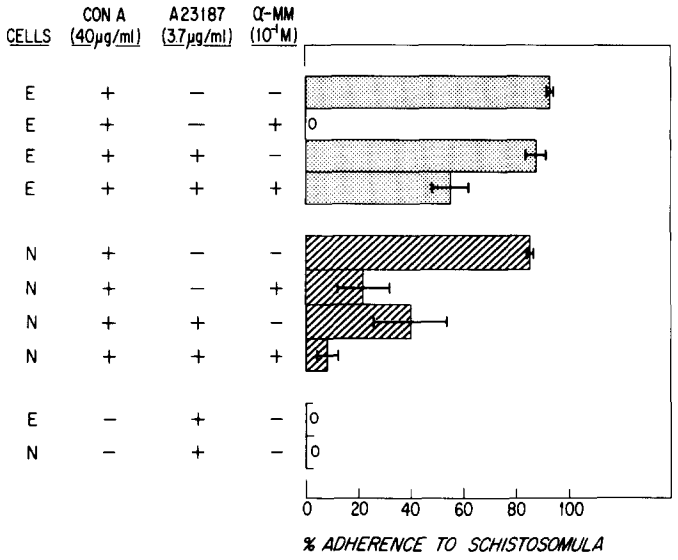

FIG. 4. Induction of degranulation with A23187 prevents the reversal of Con A-mediated eosinophil adherence induced by alpha-methylmannoside. Schistosomula were incubated either with or without Con A (40 μg/ml) and with either eosinophil-rich (83% eosinophils, 17% neutrophils) or neutrophil-rich (96% neutrophils, 4% mononuclears) cell preparations at a ratio of 2,000:1. After 1 h of incubation, A23187 was added to some groups of tubes at a final concentration of 3.7 μg/ml: medium was added to the remaining groups as controls. These preparations were then incubated for a further 1 h. Alpha-methylmannoside (10^{-1} M) was then added to some groups, with medium as control to the remainder, and the preparations were incubated for a final 1 h. Adherence was scored in duplicate tubes as the percentage of organisms bearing five or more adherent cells: mean ± 1 SD is shown.

inhibition of reversal by alpha-methylmannoside. In contrast, the complete reversal of eosinophil adherence that could be induced by alpha-methylmannoside was significantly prevented if the preparations had been treated with A23187.

A23187 Induces Killing of Schistosomula by Con A-Bound Eosinophils. Finally, it was found that induction of degranulation of Con A-bound eosinophils was indeed associated with damage to the target schistosomula. Three experiments were carried out, with comparable results, and findings from one are shown in Table IV: this experiment was the same as 4 in Table III. Schistosomula were incubated either with or without Con A, and with either eosinophil-rich or neutrophil-rich cell preparations, or with medium, for 1 h. A23187 was then added in low concentrations, and was allowed to remain present throughout the ensuing 18 h of incubation. Aliquots of the supernates of each tube were then withdrawn for the estimation of release of MBP, and the preparations were incubated for a further 24 h. At the end of this period, the numbers of dead schistosomula in each tube were scored microscopically. Additional tubes, containing anti-schistosomular serum instead of Con A and A23187, were included as a positive control.

As usual, Con A by itself did not mediate damage by either cell type, and failed to induce the release of MBP into the supernate. In contrast, the presence of anti-schistosomular serum permitted a high level of eosinophil-mediated damage, without evidence either of direct toxicity or of neutrophil-mediated damage. Detectable levels of MBP were found in the supernates of preparations containing eosinophils and anti-

schistosomular serum. Addition of A23187 induced degranulation of Con A-bound eosinophils, as reflected by the release of MBP in a dose-dependent fashion, and allowed these cells to damage the schistosomula. Damage was more marked in the presence of Con A, but was also observed at the higher concentration of ionophore in the absence of Con A. This was attributable to the formation of clumps of degranulating eosinophils: in some of these clumps, schistosomula were trapped and killed. The ionophore was not detectably toxic to the schistosomula at the concentrations tested, and failed to induce significant neutrophil-mediated damage, either with or without Con A.

Discussion

We have reported elsewhere (1)[1] that normal human eosinophils bind to schistosomula, in the presence of anti-schistosomular serum, much better than do neutrophils from the same individual. This unexpected finding was attributable to a two-stage reaction by the eosinophil. The first stage was a weak, temperature-independent interaction via the Fc receptor of the eosinophil, whereas the second stage, which was temperature-dependent, rendered this interaction stable and irreversible.

We now report that the differences observed in the ability of eosinophils and neutrophils respectively to adhere to schistosomula depend on the integrity of the target organism. When dead schistosomula were present in higher than usual numbers in the starting preparation, it was possible to compare, within a single sample, the adherence of cells to live and to dead organisms, and it was observed that neutrophils showed a greatly enhanced capacity to adhere to dead organisms. Comparable observations were made when schistosomula were damaged artificially by treatment with low concentrations of *n*-ethylmaleimide. Adherence of neutrophils to damaged schistosomula occurred in the absence of anti-schistosomular serum, but was much more marked when the antiserum was present. In such preparations, the neutrophils formed large sheaths of multiple layers of cells around the dead organisms. Similar events occur when schistosomula are damaged by cell preparations containing both eosinophils and neutrophils. Eosinophils are required for the induction of damage, because none is observed when eosinophil-depleted neutrophil preparations are tested (1): but once the organisms have been damaged, the neutrophils present in the cell mixtures may then interact with the organism. These findings imply that the neutrophil can interact with damaged schistosomula in the same way as it would with other foreign surfaces, this effect being enhanced by the presence of antischistosomular antibodies. However, when the organism is alive, the interaction of the neutrophil is much less marked, and the levels of established adherence can be markedly reduced (reversed) by the addition of agents which block the interaction between the Fc receptors and the Fc piece of IgG, such as *Staphylococcus aureus* protein A or aggregated gamma globulin.[1]

These observations may account for some of the discrepancies in the literature regarding the ability of the neutrophil to mediate primary damage to schistosomula. We have recently reported (1)[4] that although both normal human neutrophils and eosinophils can induce release of ^{51}chromium from prelabeled schistosomula in the

[4] David, J. R., M. A. Vadas, A. E. Butterworth, P. Azevedo de Brito, J. Bina, E. Cavalho, and Z. Andrade. Manuscript in preparation.

presence of antiserum, only in the case of the eosinophil is there evidence of gross damage detectable morphologically after 24 or 48 h of culture. In contrast, Anwar et al. (19) have reported that both human eosinophils and human neutrophils can induce microscopically-detectable antibody-dependent damage, and that only when complement is present is the effect of eosinophils more marked. The main difference in the techniques used is the method of preparation of the schistosomula: we have continued to use the most natural technique, namely to allow cercariae to penetrate an isolated skin preparation in vitro (17, 18), whereas Anwar et al. (19), as well as other workers (20), have used schistosomula prepared by mechanical avulsion of the cercarial tails, followed by incubation. Although these organisms are comparable in many morphological and other aspects to those prepared by skin penetration, it is clear that they differ in their ability to survive in culture (21) and to fix and to be damaged by complement (22). It may be speculated that their surfaces differ from those prepared by skin penetration, and that this may render them susceptible to persistent neutrophil adherence. This in turn may mean that an initial and transient effect, reflected in skin-prepared schistosomula by the release of ^{51}chromium, may now progress to microscopically detectable damage.

The selective ability of human eosinophils, in comparison with neutrophils, to exert progressive, microscopically detectable antibody-dependent damage to skin-prepared schistosomula is attributable, at least in part, to degranulation and release of granule contents, in particular the characteristic eosinophil MBP, onto the surface of the organism (13). MBP, in low concentrations, is toxic to schistosomula, and can be demonstrated to be released during an eosinophil-mediated reaction, both directly onto the surface of the organism and free in small amounts into the culture supernate. However, the effect of MBP itself is nonspecific, in that it can also induce damage, at comparable concentrations, to other targets (13). It was therefore of interest to examine the mechanisms whereby eosinophils may selectively adhere to antibody-coated schistosomula in a stable and irreversible fashion, in that such a selective capacity could provide a system for the delivery of the eosinophil and its contents to the relevant target. In these studies, we tested the hypothesis that selective adherence might also be attributable to degranulation, with release of granule contents onto the surface of the schistosomulum. These released granule contents might then serve as a ligand, maintaining the adherence of cells that are already attached and promoting the adherence of further cells. Two types of experiment supported this hypothesis.

First, it was found that addition of eosinophil MBP (or of protamine, a more readily available cation) enhanced the adherence of eosinophils and, to a much greater extent, of neutrophils. This effect would be predicted from the postulate that the release of MBP from eosinophil granules promotes and maintains the adherence of the eosinophil, but the observation was relatively indirect. A more direct test of the hypothesis stemmed from the observation that, although Con A would mediate high levels of adherence by both neutrophils and eosinophils, this adherence did not lead to damage with either cell type after 24 or 48 h of incubation. This unexpected finding provided us with a useful tool with which to distinguish lethal from nonlethal eosinophil adherence reactions. Because we had proposed (13) that antibody-dependent eosinophil-mediated damage was associated with degranulation and release of MBP onto the surface of the organism, an obvious first prediction was that eosinophils bound via Con A do not degranulate. In support of this, we found that such cells did

not release detectable MBP into the culture supernate, this now needs further confirmation by electron microscopy. A further prediction was that, if degranulation did not occur, then Con A-mediated eosinophil adherence should be readily reversible. This was indeed the case, and could be achieved either by washing the organisms free of excess cells and allowing them to incubate overnight, or more rapidly by competition with alpha-methylmannoside.

It was then predicted that, if Con A-bound eosinophils could now be induced to degranulate, then their adherence should no longer be readily reversible by washing and incubation or by alpha-methylmannoside, and should instead now be associated with damage to the schistosomulum. Experiments in which the calcium ionophore A23187 was used to induce degranulation confirmed this prediction, thus supporting our hypothesis that both eosinophil-mediated damage and stable eosinophil adherence are indeed dependent on degranulation. It should be emphasized that the mechanism whereby A23187 induced degranulation was not studied in these experiments: in other systems, degranulation induced by this agent is associated with a rapid influx of calcium (23–26). The actual mechanism, however, was irrelevant to the argument, because it was sufficient for the purposes of this study simply to show that degranulation had occurred. This was achieved by demonstrating an increased level of release of major basic protein into the culture supernatant (Table IV). It may also be noted that neutrophils still had no effect, even when Con A-bound cells were treated with A23187. Although we have no formal evidence that degranulation of these neutrophils had occurred, this finding would suggest that, even if neutrophils could be induced to degranulate on the surface of the schistosomulum, they would still be unable to damage the target.

Several other aspects have not been studied in these particular experiments. It will eventually be of interest to compare the antibody-dependent and the Con A-dependent reactions in terms of other known functions of the eosinophil, including hydrogen peroxide and superoxide production, in an attempt to determine whether other mechanisms, acting in concert with MBP, may play a part in mediating eosinophil damage to schistosomula. Along different lines, we have not yet examined in detail the adherence of eosinophils and neutrophils via complement instead of antibody, although preliminary data suggest that differences in adherence will be found which are comparable to those observed with anti-schistosomular sera. Finally, it will be important to compare the properties of eosinophils recovered from eosinophilic and noneosinophilic individuals. In this context, we have recently found (27)[4] that eosinophils from patients with eosinophilia are more active in mediating both ^{51}chromium release and microscopically detectable damage to schistosomula than are eosinophils from noneosinophilic subjects: and it may be speculated that this enhanced activity depends in part on a greater propensity of such cells to degranulate upon contact with an appropriate substrate.

For the moment, however, we may now assert that we are better able to understand the mode of action of the eosinophil in the simplest system, namely the reaction between normal eosinophils and schistosomula in the presence of anti-schistosomular serum. It would now appear that two factors contribute to the selective effect of the eosinophil in this situation: first, a preferential ability to adhere to the organism, and second, a preferential ability to induce damage. It would also appear that both of these factors are associated, either directly or indirectly, with eosinophil degranulation

and with the release of granule components, in particular the characteristic MBP, onto the surface of the organism. In this respect, the eosinophil may now be regarded as a highly specialized cell, one of whose major properties may be to interact with and damage large, nonphagocytosable particles such as tissue-stage helminths.

Summary

Previous work (1)[1] has shown that normal human eosinophils show a preferential capacity, in comparison with neutrophils, to bind to antibody-coated schistosomula of *Schistosoma mansoni*. This effect is attributable to a temperature-dependent function of the eosinophil which renders its binding stable and irreversible by aggregated gamma globulin or *Staphylococcus aureus* protein A. In contrast, the binding of neutrophils is readily reversible by these agents.

It has now been shown that the differences observed between eosinophils and neutrophils is a property of their interaction with living schistosomula. When dead or artificially damaged schistosomula were tested, neutrophils showed a markedly enhanced capacity to adhere, in both the presence and absence of anti-schistosomular serum.

Subsequent experiments were designed to test the hypothesis that the strong, stable binding of eosinophils was attributable to degranulation, with release of granule contents which would then serve as ligands to bind the cell to the organism. First, an enhanced adherence both of eosinophils and of neutrophils could be demonstrated in the presence of eosinophil major basic protein (MBP) or of protamine, a high molecular weight cation. Second, the binding of eosinophils induced by concanavalin A (Con A) was found to differ markedly from that induced by antischistosomular serum. Con A-mediated binding of eosinophils was fully reversible by alpha-methylmannoside, was not associated with damage to the organism, and did not lead to degranulation of the cell, as estimated by measuring the release of MBP into the culture supernate. However, induction of degranulation of concanavalin A-bound eosinophils, but not of neutrophils, with the calcium ionophore A23187 converted the reaction into one which was no longer reversible by alpha-methylmannoside and in which damage to the organism now did occur.

These findings support the hypothesis that the stable binding of eosinophils is associated with degranulation, a process which may contribute to the preferential capacity of this cell to mediate antibody-dependent damage to schistosomula.

We are deeply grateful to Dr. Eric Martz for his invaluable and constructive comments at all stages of the study. We thank D. A. Loegering for his capable technical assistance.

Received for publication 28 June 1979.

References

1. Vadas, M. A., J. R. David, A. E. Butterworth, N. T. Pisani, and T. A. Siongok. 1979. A new method for the purification of human eosinophils and neutrophils, and a comparison of the ability of these cells to damage schistosomula of *Schistosoma mansoni*. *J. Immunol.* **122:** 1228.
2. Tai, P. E., and C. J. F. Spry. 1976. Studies on blood eosinophils. I. Patients with a transient eosinophilia. *Clin. Exp. Immunol.* **24:**415.
3. Gupta, S., G. D. Ross, R. A. Good, and F. P. Siegal. 1976. Surface markers of human eosinophils. *Blood.* **48:**755.

4. Ottesen, E. A., A. M. Stanley, J. A. Gelfand, J. E. Gadek, M. M. Frank, T. E. Nash, and A. W. Cheever. 1977. Immunoglobulin and complement receptors on human eosinophils and their role in cellular adherence to schistosomules. *Am. J. Trop. Med. Hyg.* **26** (Suppl. 6): 134.
5. Anwar, A. R. E., and A. B. Kay. 1977. Membrane receptors for IgG and complement (C4, C3b and C3d) on human eosinophils and neutrophils and their relation to eosinophilia. *J. Immunol.* **119:**976.
6. Glauert, A. M., and A. E. Butterworth. 1977. Morphological studies on antibody-dependent, eosinophil-mediated cytotoxicity to schistosomula of *Schistosoma mansoni*. *Trans. R. Soc. Trop. Med. Hyg.* **71:**291.
7. Glauert, A. M., and A. E. Butterworth. 1977. Morphological evidence for the ability of eosinophils to damage antibody-coated schistosomula. *Trans. R. Soc. Trop. Med. Hyg.* **71:**392.
8. Glauert, A. M., A. E. Butterworth, R. F. Sturrock, and V. Houba. 1978. The mechanism of antibody-dependent, eosinophil-mediated damage to schistosomula of *Schistosoma mansoni* in vitro: a study by phase-contrast and electron microscopy. *J. Cell. Sci.* **34:**173.
9. McClaren, D. J., C. D. Mackenzie, and F. J. Ramalho-Pinto. 1977. Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (*Schistosoma mansoni*, *Trichinella spiralis* and *Nippostrongylus braziliensis*). *Clin. Exp. Immunol.* **30:**105.
10. McClaren, D. J., F. J. Ramalho-Pinto, and S. R. Smithers. 1978. Ultrastructural evidence for complement and antibody-dependent damage to schistosomula of *Schistosoma mansoni* by rat eosinophils in vitro. *Parasitology.* **77:**313.
11. Gleich, G. J., D. A. Loegering, and J. E. Maldonado. 1973. Identification of a major basic protein in guinea pig eosinophil granules. *J. Exp. Med.* **137:**1459.
12. Gleich, G. J., D. A. Loegering, F. Kueppers, S. P. Bajaj, and K. G. Mann. 1974. Physicochemical and biological properties of the major basic protein from guinea pig eosinophil granules. *J. Exp. Med.* **140:**313.
13. Butterworth, A. E., D. L. Wassom, G. J. Gleich, D. A. Loegering, and J. R. David. 1979. Damage to schistosomula of *Schistosoma mansoni* induced directly by eosinophil major basic protein. *J. Immunol.* **122:**221.
14. Caulfield, J. P., A. E. Butterworth, G. Korman, and J. R. David. 1978. Antibody- and complement-mediated junction formation between *Schistosoma mansoni* and polymorphonuclear leukocytes, macrophages, and erythrocytes. *J. Cell Biol.* **79**(2):92A.
15. Gleich, G. J., D. A. Loegering, K. G. Mann, and J. E. Maldonado. 1976. Comparative properties of the Charcot-Leyden crystal protein and the major basic protein from human eosinophils. *J. Clin. Invest.* **57:**633.
16. Wassom, D. L., D. A. Loegering, and G. J. Gleich. 1979. Measurement of guinea-pig eosinophil major basic protein by radioimmunoassay. *Mol. Immunol.* In press.
17. Stirewalt, M. A., D. R. Minnick, and W. A. Fregeau. 1966. Definition and collection in quantity of schistosomules of *Schistosoma mansoni*. *Trans. R. Soc. Trop. Med. Hyg.* **60:**352.
18. Clegg, J. A., and S. R. Smithers. 1972. The effects of immune Rhesus monkey serum on schistosomula of *Schistosoma mansoni* during cultivation in vitro. *Int. J. Parasitol.* **2:**79.
19. Anwar, A. R. E., S. R. Smithers, and A. B. Kay. 1979. Killing of schistosomula of *Schistosoma mansoni* coated with antibody and/or complement by human leukocytes in vitro: requirement for complement in preferential killing by eosinophils. *J. Immunol.* **122:**628.
20. Ramalho-Pinto, F. J., D. J. McClaren, and S. R. Smithers. 1978. Complement-mediated killing of *Schistosoma mansoni* by rat eosinophils in vitro. *J. Exp. Med.* **147:**147.
21. Brink, L. H., D. J. McClaren, and S. R. Smithers. 1977. *Schistosoma mansoni:* a comparative study of artificially transformed schistosomula and schistosomula recovered after cercarial penetration of isolated skin. *Parasitology.* **74:**73.
22. Santoro, F., P. J. Lachmann, A. Capron, and M. Capron. 1979. Activation of complement

by *Schistosoma mansoni* schistosomula. Killing of parasites by the alternative pathway and requirement of IgG for the classical pathway activation. *J. Immunol.* In press.

23. Goldstein, I. M., J. K. Korn, H. B. Kaplan, and G. Weissmann. 1974. Calcium-induced lysozyme secretion from human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **60:**804.
24. Showell, H. J., P. H. Naccache, R. I. Sha'afi, and E. L. Becker. 1977. Effect of extracellular Ca^{+2}, Na^{+}, K^{+} and La^{+3} on lysosomal enzyme release by rabbit neutrophils. *J. Immunol.* **119:**804.
25. Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1977. Changes in ionic movements across rabbit polymorphonuclear leukocyte membranes during lysosomal enzyme release. *J. Cell Biol.* **75:**635.
26. Foreman, J. C., M. B. Hallett, and J. L. Mongar. 1977. The relationship between histamine secretion and ^{45}calcium uptake by mast cells. *J. Physiol.* **271:**193.
27. Vadas, M. A., J. R. David, A. E. Butterworth, V. Houba, R. F. Sturrock, L. David, R. Henson, T. A. Siongok, and R. Kimani. Functional studies on purified eosinophils and neutrophils from patients with *Schistosoma mansoni* infection. *Clin. Exp. Immunol.* In press.