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The Bentonite Granuloma

CHARACTERIZATION OF A MODEL SYSTEM FOR INFECTIOUS AND FOREIGN BODY GRANULOMATOUS INFLAMMATION USING SOLUBLE MYCOBACTERIAL, HISTOPLASMA AND SCHISTOSOMA ANTIGENS

D. L. Boros and K. S. Warren

Departments of Medicine and Community Health (Preventive Medicine), Case Western Reserve University School of Medicine and University Hospitals, Cleveland, Ohio 44106, *U.S.A.*

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Summary. A model for the study of granulomatous inflammation has been developed, employing bentonite particles (65 μm in diameter) coated with soluble antigens derived from *Mycobacterium tuberculosis*, *Histoplasma capsulatum* and *Schistosoma mansoni*. These particles are injected i.v. into the micro-vasculature of the lungs of unsensitized mice or mice sensitized to the above antigens in a variety of ways. Uncoated particles or those coated with heterologous antigens elicit rapidly forming small foci of inflammatory cells composed mainly of macrophages. In contrast, particles coated with homologous antigens elicit large hypersensitivity-type granulomas composed of lymphocytes, macrophages, eosinophils and occasionally epithelioid cells. The occurrence of hypersensitivity-type granulomas correlates well with delayed footpad swelling also elicited by homologous antigen. In addition, the florid specific granulomatous inflammation is transferrable to syngeneic recipients with immune lymphoid cells but not with antiserum. Finally, when bare or heterologous antigen-coated particles are injected into sensitized mice which are simultaneously injected i.v. with homologous antigen, hypersensitivity granulomas appear around the particles. This bentonite model thus facilitates the study of both the foreign body and hypersensitivity granulomas and suggests mechanisms by which they might be formed.

INTRODUCTION

Many major diseases (e.g. tuberculosis, leprosy, histoplasmosis, schistosomiasis) are characterized by chronic granulomatous inflammation. Investigation of the mechanism of the granulomatous process in these diseases has been hampered, however, by the small size of most of the organisms, their intracellular habitat, and the fact that they multiply and disseminate in the infected host. While many intracellular bacteria and fungi induce a state of delayed hypersensitivity in the host, often concomitant with the appearance of acquired antimicrobial resistance (Mackaness and Blanden, 1967), the relationship between these immunological mechanisms and granuloma formation is still undefined.

Recently, studies of the schistosome egg granuloma have provided much information on the etiology of granulomatous hypersensitivity. The schistosome egg is large (150 × 60 μm), contains a living, antigen-secreting embryo protected by an eggshell, and neither replicates nor disseminates in the mammalian host. Schistosome eggs may be isolated and injected i.v. into mice. They lodge in the microvasculature of the lungs and, as individual nidi, elicit measurable inflammatory reactions. Using this system it was demonstrated that granuloma formation was both accelerated and augmented after previous exposure to eggs, that these reactions were specific, and that they could be transferred with immune lymphoid cells but not with antiserum (Warren, Domingo and Cowan, 1967).

Soluble antigens were then isolated from the eggs which induced granulomatous and dermal hypersensitivity in mice (Boros and Warren, 1970) and guinea-pigs, and also elicited blast transformation and macrophage migration inhibition in the latter (Boros, Schwartz, Warren, and Seabury, 1971). Adsorption of the soluble egg antigens onto bentonite (a colloidal aluminum silicate) approximately the size of the schistosome eggs provided a fixed antigenic focus which elicited granulomatous inflammation after injection into sensitized mice (Boros and Warren, 1970). Since the reactions resembled the infectious granuloma both in size and cellular composition, it was concluded that this model simulated the conditions existing during the actual infection. This model has made it possible for the first time to study the granuloma-eliciting properties of soluble antigens obtained from replicating organisms as described briefly in a preliminary report (Boros and Warren, 1971).

In the present investigation, the bentonite granuloma system was characterized further using soluble schistosome egg, mycobacterial, and fungal antigens. Animals were infected with *Schistosoma mansoni*, *Mycobacterium tuberculosis* or *Histoplasma capsulatum* or were sensitized by a variety of antigens obtained from these organisms. (Most of these animals displayed delayed footpad swelling on injection of the homologous antigens.) When bentonite particles coated with homologous antigens were injected into these animals, they elicited large infectious granulomas. In contrast, uncoated particles or those coated with heterologous antigens resulted in small foreign body granulomas. Infectious granulomatous reactivity was transferred by immune lymphoid cells but not by serum. Finally, it was shown that infectious type granulomas would form around either bare particles or particles coated with heterologous antigens if antigen-adjuvant sensitized or infected animals received systemic injections of homologous antigen concomitantly with the particles.

On the basis of the above observations, the bentonite granuloma is proposed as a model for exploring the mechanisms underlying the formation of not only the infectious granuloma and the foreign body granuloma, but those of unknown etiology as well. The system also may be useful for the characterization of soluble antigens involved in granulomatous diseases.

MATERIALS AND METHODS

ANIMALS

Two strains of female mice were employed throughout the experiments: Swiss albino CF1 (Carworth Farms, New City, N.Y.), and C3H/HeJ (inbred) (Jackson Laboratories, Bar Harbor, Maine). All mice weighed 18–20 g at the onset of the experiments.

INFECTION AND SENSITIZATION OF ANIMALS

A. Organisms

M. tuberculosis, strain $H_{37}Ra$, came from the stock collection of the Institute of Pathology, Case Western Reserve University School of Medicine, where it had been maintained on Lowenstein–Jensen medium (Difco). Mycobacteria were inoculated into Dubos broth base liquid medium containing Tween 80 (Difco) and were cultured for 2½ weeks at 37°.

H. capsulatum, strain S-232, had been maintained as a yeast phase on Kurung egg medium at 4°. Cultures were prepared by inoculating the organisms into brain heart infusion with 2 per cent agar (Difco) and incubating at 37° for 48 hours; the organisms were then subcultured on fresh slants for an additional 48 hours. After this, the yeast cells were washed off with sterile saline and were kept in suspension until inoculated.

S. mansoni (Puerto Rican strain) was maintained in infected *Biomphalaria glabrata* snails in our laboratory.

B. Antigens

For induction of sensitization, elicitation of dermal reactions and coating of bentonite particles, soluble antigens were used in all three infectious models:

1. *Mycobacterial* antigen. A ten-fold concentrate of the culture filtrate of *M. tuberculosis* strain $H_{37}Ra$ was used. Preparation of this mycobacterial culture filtrate (MCF) and its antigenic properties were described by Daniel and Ferguson (1970). This material contained 5 mg protein per ml.

2. *Histoplasma* antigen. Histoplasma culture filtrate (HCF) was prepared by growing mycelial colonies of *H. capsulatum* strain S-76 in an asparagine synthetic medium. This protein-free medium consisted of: asparagine, 7·0 g; ammonium chloride, 7·0 g; $K_2HPO_4 \cdot 5H_2O$, 1·31 g; sodium citrate ($5\frac{1}{2}$ H_2O), 0·9 g; $Mg\ SO_4 \cdot 7H_2O$, 1·5 g; ferric citrate, 0·3 g; dextrose, 10·0 g; glycerine, 25·0 g; and distilled water to make up to 1000 ml. Cultures were incubated in 1000 ml Erlenmeyer flasks without agitation at 23° for 7 months. At the end of the incubation period, broth was aspirated and Seitz filtered. This filtrate contained 1·5 mg protein per ml.

3. *Schistosoma* antigens. Soluble egg antigens (SEA) were obtained from homogenized ultracentrifuged *S. mansoni* eggs as previously described (Boros and Warren, 1970). The material contained 0·5 mg protein per ml.

Protein determinations of the antigens were made according to the method of Lowry (Lowry, Rosebrough, Farr and Randall, 1951). As standard, crystalline bovine serum albumin (Pentex, Kankakee, Illinois) was used. Particulate antigens were also used to sensitize some groups of mice. These included formalin killed yeast cells (FYC) as prepared by Hill and Campbell (1956) and viable schistosome eggs which were isolated and purified as described earlier (Warren *et al.*, 1967).

C. Infection of mice

Animals were infected i.p. with a 2½-week-old culture of tubercle bacilli, as described by Dietrich, Nordin and Bloch (1962). Three weeks later the infected state was verified by homogenizing spleens and plating a drop of homogenate on Lowenstein–Jensen medium. Tubercle bacilli were cultured from all homogenates.

Live yeast cell suspensions of *H. capsulatum* were counted in a haemocytometer and standardized to 500,000 cells per 0·5 ml suspension. Mice were inoculated i.p. with 0·5 ml of the suspension. Three weeks later, the infected state was verified by homogenizing spleens and plating a drop of homogenate on Sabouraud's Dextrose Agar medium (Difco). Yeast cells were cultured from all homogenates.

Infection with schistosomes was done by the s.c. injection of 150 cercariae into mice according to the method of Peters and Warren (1969). Eight weeks later the infected state was verified by gross examination of livers which showed numerous granulomatous foci and fibrosis. All the inoculated animals were infected.

D. Sensitization of mice

This was done by the i.p. or s.c. injection of soluble or particulate antigens either alone or mixed with Freund's complete adjuvant (FCA) containing 10 mg/ml *M. tuberculosis* strain $H_{37}Ra$ (Difco).

One group of mice received 500 μg MCF i.p. in 0·1 ml volume, while another received the same amount of MCF incorporated into an equal volume of FCA and injected s.c. into the loose skin of the back.

Groups of mice received 150 μg HCF i.p. in 0·1 ml volume, or 0·1 ml of formalin killed yeast cells of *H. capsulatum* (FYC) containing 5×10^6 ml cells. A third group of mice received the same amount of FYC incorporated into equal volumes of FCA injected s.c.

Additional groups of mice were injected i.p. with 50 μg of SEA in 0·1 ml, or the same amount of antigen was incorporated into FCA and injected s.c. as described above. Another group of mice was injected i.p. with 5000 viable schistosome eggs suspended in saline.

A final group received an i.p. injection of 25,000 washed bentonite particles suspended in 0·5 ml saline.

Duration of sensitization was 14 days in all modes of immunization.

ELICITATION OF GRANULOMA FORMATION WITH SOLUBLE ANTIGENS ADSORBED TO BENTONITE PARTICLES

The source and standardization of bentonite particles, as well as the method of coating, were described previously (Boros and Warren, 1970). Five to 6000 particles either bare or coated with MCF, HCF or SEA were injected i.v. Four days after injection mice were killed, their lungs filled with buffered formalin and removed. Preparation of histological sections and granuloma measurements were done as described previously (Boros and Warren, 1970).

DELAYED FOOTPAD SWELLING

Dermal reactivity of infected or sensitized mice was examined by the footpad reaction. For elicitation of the reaction, 0·03 ml of the different soluble antigens containing 30 μg protein was injected. The method of injection and measurement of swelling were described earlier (Boros and Warren, 1970). Reactions were measured at 3, 6, and 24 hours after the injection. At the appropriate time periods, the hind feet of challenged animals were

amputated and fixed in 10 per cent buffered formalin. Decalcification was performed by immersion in 30 per cent formic acid solution containing 10 per cent Win-3000 granular resin. Sections were prepared and stained with haematoxylin and eosin.

PASSIVE TRANSFER OF LYMPHOID CELLS AND IMMUNE SERUM

A. Cells

For passive transfer of cells, spleens and mesenteric lymph nodes of infected and adjuvant-sensitized syngeneic C3H mice were used. Mice were anaesthesized with ether, the neck vein cut, and the blood collected by a Pasteur pipette. The abdomen was washed with alcohol and opened, and spleens and lymph nodes were removed using sterile instruments. The organs were minced with a pair of scissors, and the fragments were gently strained through a stainless steel wire screen (325 mesh size) by a pestle. Cell suspensions were prepared using Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) with the addition of 10 per cent foetal calf serum. Suspended cells were washed twice by centrifugation (1000 rev/min for 6 minutes) then resuspended in 0·7 ml volume of the same medium and counted in a haemocytometer. Viability, checked by the addition of trypan blue, was usually greater than 95 per cent. Lymphoid cells of each donor were separately processed and injected into each recipient, the average number of cells transferred being $1{\cdot}6 \times 10^8$. Lymphoid cell suspensions of the histoplasma- and mycobacterium-infected animals were plated on plastic 25 cm^2 tissue culture flasks (Falcon Plastics, Los Angeles, Ca.) in order to decrease the number of macrophages and the chance of transfer of intracellular organisms. Bottles containing 2 ml cell suspensions were gassed with a mixture of 95 per cent O_2 and 5 per cent CO_2, tightly closed, and incubated for 3 hours at 37°. After incubation the cells were aspirated and the bottles were gently rinsed so as not to disturb the adhering cells. This treatment resulted in approximately a 30 per cent loss in cell numbers. Three days after cell transfer, recipients in parallel groups were challenged with coated bentonite particles injected into the microvasculature of the lungs, or with soluble antigens injected into the footpad. Normal lymphoid cells were similarly prepared for transfer.

B. Serum

Sera were collected and pooled from the blood of infected and antigen-adjuvant sensitized syngeneic mice, and recipient mice were given a total of 1·2 ml serum per animal on the following schedule: Mice being injected with coated bentonite particles received 0·3 ml at intervals of 2 days before, 1 day before, 30 minutes before, and 2 days after the injection of particles. Mice being tested for footpad swelling received 0·3 ml 2 days before, 0·3 ml 1 day before, and 0·6 ml 30 minutes before the footpad injection. Normal pooled serum was injected according to a similar schedule.

SEROLOGICAL DETERMINATIONS OF ANTIBODY TITRES

The antibody content of pooled schistosome serum was assayed by the passive haemagglutination method according to Boyden (1951). One millilitre of 2·5 per cent sheep erythrocytes was mixed with 1 ml of SEA (500 μg protein). Heteroagglutinins were absorbed from the serum with washed sheep erythrocytes before the test. Titration was done using the micro-plate method. Titre of serum was 1 : 320.

Pooled histoplasma serum was examined by complement fixation according to Hill and Campbell (1956). As antigen, the yeast phase of histoplasma was used. Titre was 1:32.

Pooled mycobacterium serum was tested by the haemagglutination method of Middlebrook-Dubos (1948), as modified by Daniel (1965). Titre was 1:1280.

GRANULOMA FORMATION DURING CONCOMITANT SYSTEMIC ANTIGENIC STIMULUS

Infected or sensitized mice received four consecutive daily i.v. injections of 100 μg of soluble antigens (SEA, MCF or HCF) beginning 30 minutes before injection of bentonite particles and continuing daily for 3 days. Control animals were injected i.v. with phosphate buffered saline (PBS). Lungs were removed 4 days after the bentonite injection.

RESULTS

GRANULOMA FORMATION AROUND ANTIGEN-COATED PARTICLES IN MICE SENSITIZED BY DIFFERENT ROUTES AND ANTIGENS

In order to define the conditions under which the hypersensitivity type bentonite granuloma is formed, groups of mice were sensitized with soluble or particulate antigens, with or without the aid of complete adjuvant, or were experimentally infected. The variously sensitized animals were challenged with bentonite particles coated with the homologous soluble antigens. Table 1 shows granuloma sizes as measured in stained lung sections. As can be seen, significantly larger ($P<0{\cdot}005$) granulomas, as compared to controls (coated or uncoated bentonite in lungs of normal mice), appeared only in

TABLE 1

GRANULOMA FORMATION AROUND ANTIGEN-COATED BENTONITE PARTICLES (MEAN DIAMETER—65 μm) 4 DAYS AFTER THEIR i.v. INJECTION INTO THE LUNGS OF SENSITIZED MICE

Mode of sensitization	Bentonite alone	Bentonite coated with soluble antigens of:		
		M. tuberculosis	*H. capsulatum*	*S. mansoni*
		Mean granuloma diameter, μm $\pm$ SE		
M. tuberculosis				
MCF, i.p.		66 $\pm$ 2 (6/100)*		
MCF + FCA, s.c.		103 $\pm$ 4 (6/ 74)		
Infected		96 $\pm$ 3 (4/ 59)		
H. capsulatum				
HCF, i.p.			62 $\pm$ 3 (6/78)	
FYC, i.p.			69 $\pm$ 2 (6/80)	
FYC + FCA, s.c.			113 $\pm$ 3 (6/64)	
Infected			105 $\pm$ 3 (6/82)	
S. mansoni				
Eggs, i.p.				132 $\pm$ 5 (6/ 88)
SEA, i.p.				105 $\pm$ 4 (6/100)
SEA + FCA, s.c.				139 $\pm$ 4 (5/ 67)
Infected				103 $\pm$ 4 (6/100)
Control				
Unsensitized	69 $\pm$ 2 (6/ 66)	70 $\pm$ 3 (3/ 45)	72 $\pm$ 4 (5/62)	77 $\pm$ 3 (5/ 80)
'Sensitized' (bentonite, i.p.)	66 $\pm$ 2 (5/100)			

* Number of mice/number of lesions measured.

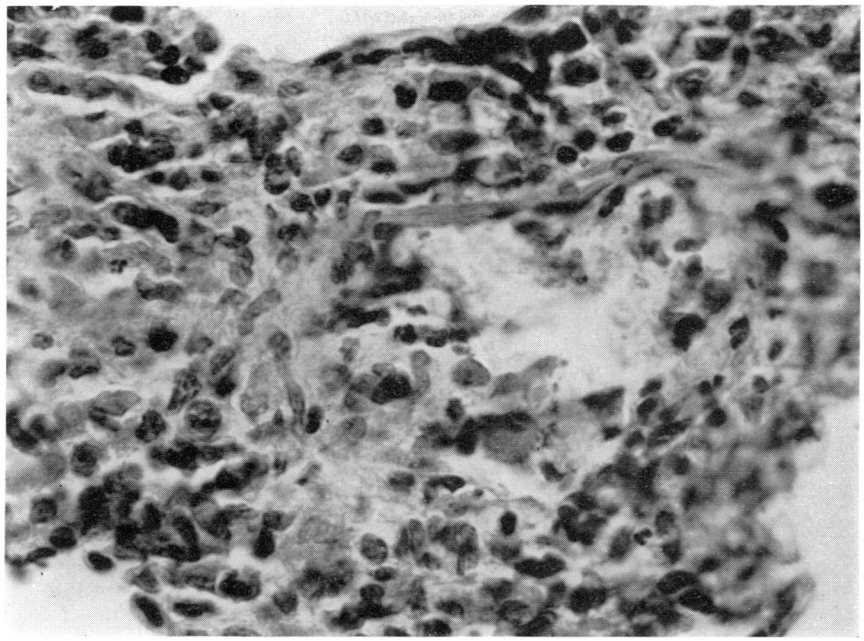

FIG. 1. Granuloma formation at 4 days around an MCF-coated bentonite particle in the lung of a mycobacterium-infected mouse. Haematoxylin and eosin stain. (× 720.)

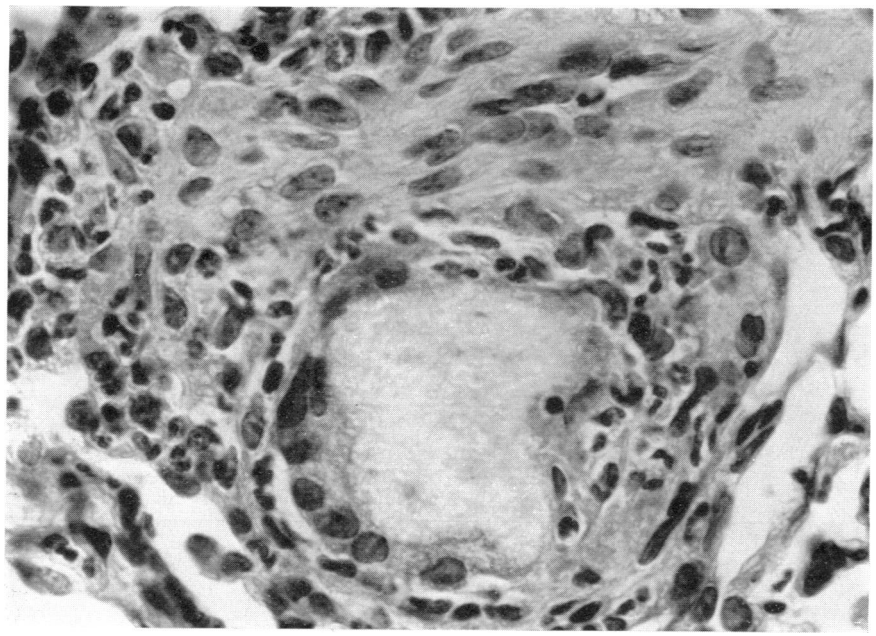

FIG. 2. Granuloma formation at 4 days around an SEA-coated bentonite particle in the lung of a schistosome-infected mouse. Haematoxylin and eosin stain. (× 720.)

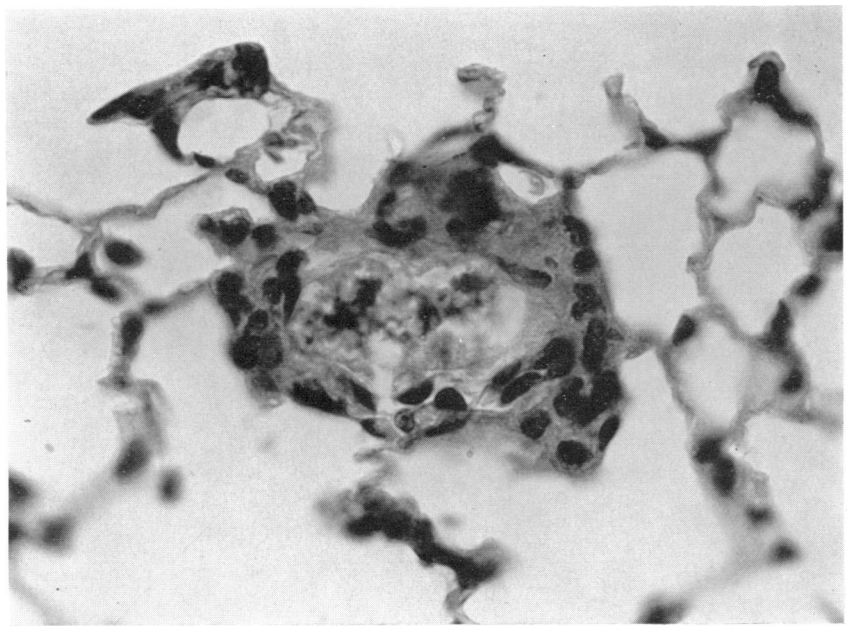

FIG. 3. Cellular reaction at 4 days around a bare bentonite particle in the lung of an unsensitized mouse. Haematoxylin and eosin stain. (× 720.)

antigen-adjuvant sensitized or infected animals. In accordance with earlier observations (Boros and Warren, 1970), i.p. injection of schistosome eggs or SEA also sensitized mice to a subsequent enhanced granulomatous reaction around the coated particles. These large lesions resembled the so-called hypersensitivity, allergic, or infectious granuloma in that they were composed of macrophages, lymphocytes, eosinophils and occasionally epithelioid cells (Figs 1 and 2).

In contrast, uncoated particles in normal animals evoked a scanty cellular reaction composed mainly of macrophages (Fig. 3), a picture consistent with that of the foreign body granuloma. It is noteworthy that bentonite particles themselves were immunologically inert, as after the i.p. injection of large numbers of particles, the reactions

TABLE 2

GRANULOMA FORMATION AROUND BENTONITE PARTICLES COATED WITH HOMOLOGOUS OR HETEROLOGOUS ANTIGENS INJECTED I.V. INTO THE LUNGS OF SENSITIZED MICE

Mode of sensitization	Bentonite coated with soluble antigens of:		
	M. tuberculosis	*H. capsulatum*	*S. mansoni*
	Mean granuloma diameter, $\mu m \pm SE$		
MCF + FCA, s.c.	104 ± 4 (6/77)*	78 ± 3 (5/59)	71 ± 3 (6/87)
FYC + FCA, s.c.	N.D.†	113 ± 3 (6/64)	79 ± 5 (3/32)
Schistosoma eggs, i.p.	73 ± 4 (6/92)	69 ± 4 (3/28)	132 ± 5 (6/88)

* Number of mice/number of lesions measured.
† Not done as FCA contains Mycobacteria.

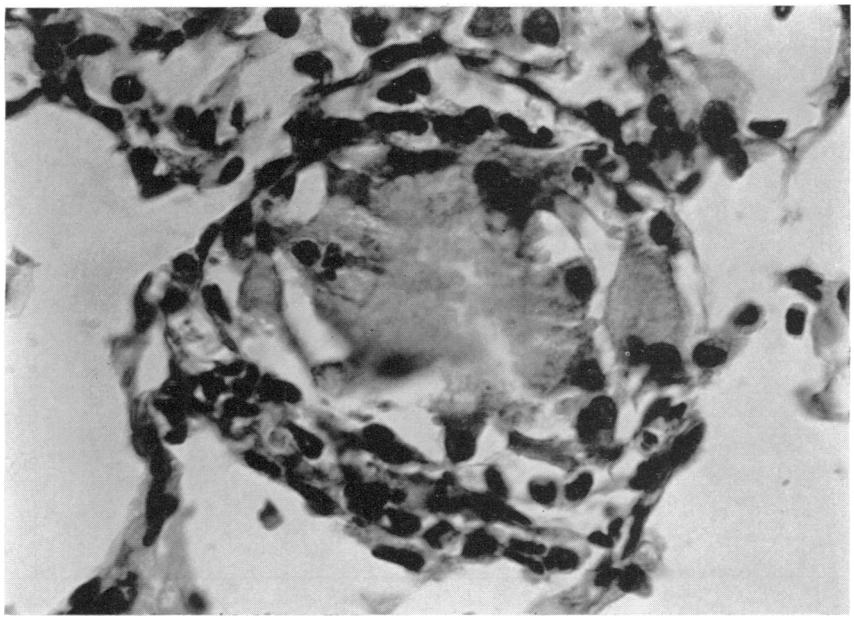

FIG. 4. Cellular reaction at 4 days around MCF-coated bentonite particle in the lung of schistosome-infected mouse. Haematoxylin and eosin stain. (× 720.)

around particles subsequently injected into the lungs were identical to those seen in animals previously unexposed to bentonite.

THE SPECIFICITY OF THE BENTONITE GRANULOMA

The immunological nature of the bentonite granuloma was further verified by showing that infectious type granulomas in sensitized mice were formed only around particles coated with the homologous antigen (Table 2). Lack of cross-reactivity was demonstrated by the scanty, foreign body type reaction around the particles coated with heterologous antigens (Fig. 4). In our investigations of specificity in which the cross-sensitizing characteristics of the various antigens were studied, the unique properties of SEA provided a great advantage. Mice sensitized with viable eggs or SEA alone, without the addition of oil or mycobacteria, developed large infectious type granulomas around particles coated with homologous antigen. Thus it was shown that the presence of the ingredients of complete adjuvant was not necessary for the induction of the granulomatous response.

ADOPTIVE TRANSFER OF INFECTIOUS GRANULOMA FORMATION AROUND ANTIGEN-COATED BENTONITE PARTICLES BY LYMPHOID CELLS AND SERUM

In the previous experiments it was shown that enhanced hypersensitivity-type granuloma formation is specific and occurs (with the exception of the schistosome system) only in adjuvant-sensitized or infected animals. In a further series of experiments, it could be shown that granuloma formation is transferrable by lymphoid cells obtained from the

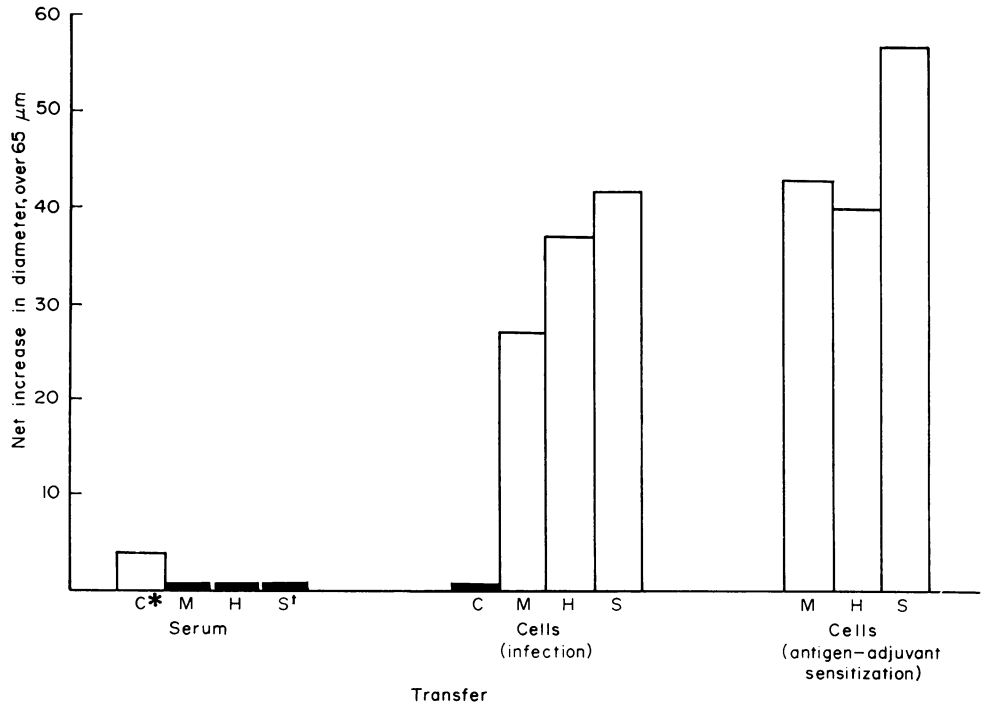

FIG. 5. Granuloma formation around antigen-coated bentonite particles in lungs of recipients of immune serum or cells. Each bar represents the mean results of five or more mice. * Control, normal mice. † M = mycobacterium; H = histoplasma; S = schistosoma.

sensitized or infected donors (Fig. 5). The cells of the infected donors were slightly less effective than those of the antigen-adjuvant immune animals. Normal lymphoid cells were ineffective in the adoptive transfer.

In contrast to the enhanced granulomatous reactions seen in recipients adoptively immunized with immune lymphoid cells, mice which received a total of 1·2 ml antiserum reacted with small foreign-body type granulomas around coated bentonite particles. Granuloma sizes were similar to those of control, normal serum-injected mice. In the adoptive transfer experiments recipients of sensitized cells were challenged 3 days after transfer. Since it was recognized that a remote possibility existed for an active sensitization by transferred antigens, the following experiments were done in the mycobacterium system: (1) Sensitized lymphoid cells were transferred into recipients according to the regular protocol. Four of these mice were immediately challenged with coated particles. Granuloma sizes at 4 days were identical with those seen in animals challenged 3 days after transfer: 110 ± 5 μm. (2) A group of four mice received sensitized lymphoid cells lysed with distilled water before transfer. Three days later recipients were challenged with the antigen-coated particles. Granuloma sizes at 4 days were minimal: 68 ± 2 μm, showing that lysed cells were incapable of transferring sensitivity.

FOOTPAD REACTIVITY

A. In mice sensitized by different routes and antigens

In parallel with the challenge injections of coated bentonite particles, groups of mice

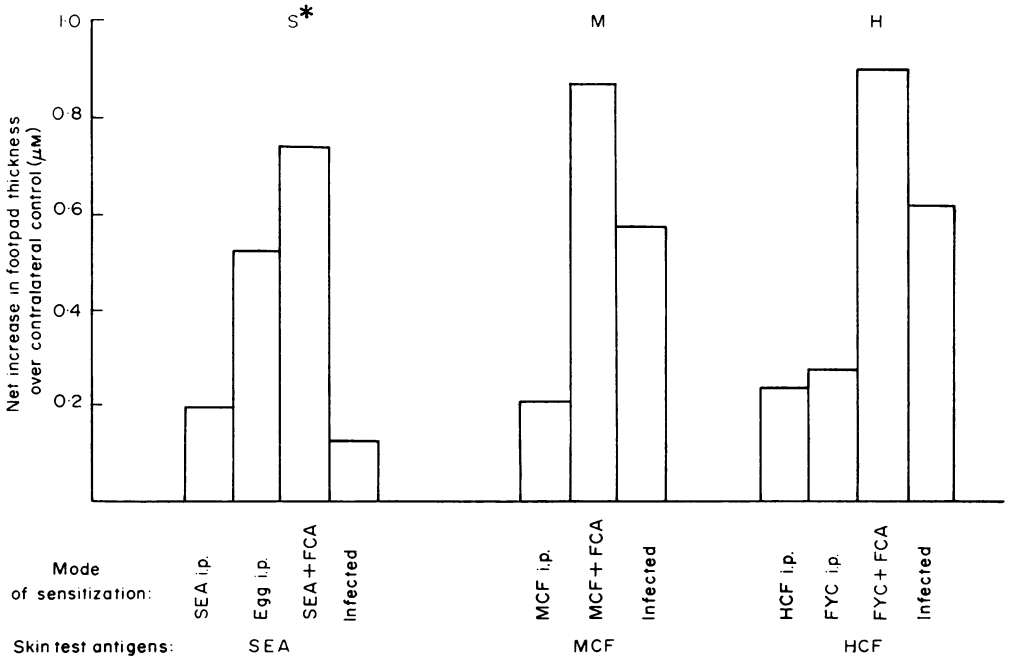

FIG. 6. Delayed footpad swelling (24 hours) in mice sensitized to mycobacteria, histoplasma or schistosoma. Each bar represents the mean results of five mice. * S = schistosome system; M = mycobacterium system; H = histoplasma system.

sensitized by a variety of means were skin tested with the soluble antigens employed for the coating of particles. As can be seen (Fig. 6), the state of immunization was also reflected in the dermal reactivity of the animals. At 24 hours significant delayed swelling (more than a 15 per cent increase, as compared with the contralateral pad) was seen only in infected

TABLE 3

FOOTPAD SWELLING FOLLOWING THE INJECTION OF HOMOLOGOUS ANTIGENS INTO RECIPIENTS OF SERUM OR LYMPHOID CELLS FROM MICE WITH TUBERCULOSIS, HISTOPLASMOSIS OR SCHISTOSOMIASIS

Recipients of:		Mean increase in footpad thickness* at intervals after injection of soluble antigens		
		3 hours	6 hours mm ± SE	24 hours
Mycobacterium-immune	A. Serum†	0·67 ± 0·08	0·46 ± 0·07	0·08 ± 0·02
	B. Cells	0·77 ± 0·08	0·79 ± 0·08	0·31 ± 0·03
Histoplasma-immune	A. Serum	0·18 ± 0·03	0·12 ± 0·02	0·03 ± 0·02
	B. Cells	0·88 ± 0·06	0·64 ± 0·06	0·36 ± 0·03
Schistosoma-immune	A. Serum	0·49‡ ± 0·02	0·22 ± 0·03	0·04 ± 0·01
	B. Cells	0·24 ± 0·02	0·18 ± 0·03	0·12 ± 0·05

* Right paw thickness (antigens) minus left paw thickness (saline) (mean difference between right and left pads before injection averaged: 0·03 mm).

† Serum derived from infected and antigen-adjuvant sensitized donors. Cells from infected donors only.

‡ Each number represents the mean results of five mice.

or antigen-adjuvant sensitized animals. An exception occurred in the schistosome system where egg sensitized mice displayed good delayed dermal reactivity, while infected mice showed relatively small footpad reactions at 24 hours. When compared with Table 1, a correlation can be seen between enhanced granuloma formation and delayed type dermal reactivity in the differently immunized mice.

B. In mice following passive transfer of cells and serum

Parallel groups of recipients of serum or cells were challenged in the footpad with the homologous antigens. Footpad swelling was recorded at 3, 6 and 24 hours after injection. As Table 3 shows, recipients of antiserum displayed a typical immediate type of dermal reaction peaking at 3 hours and subsequently declining until, at 24 hours, virtually no swelling remained. Recipients of immune cells displayed significant delayed reactions, but these were only 30–60 per cent of the size of the actively immunized animals. Schistosome immune cells induced particularly weak reactivity. Peak reactivity in recipients of mycobacterium immune cells consistently occurred between 6 and 24 hours.

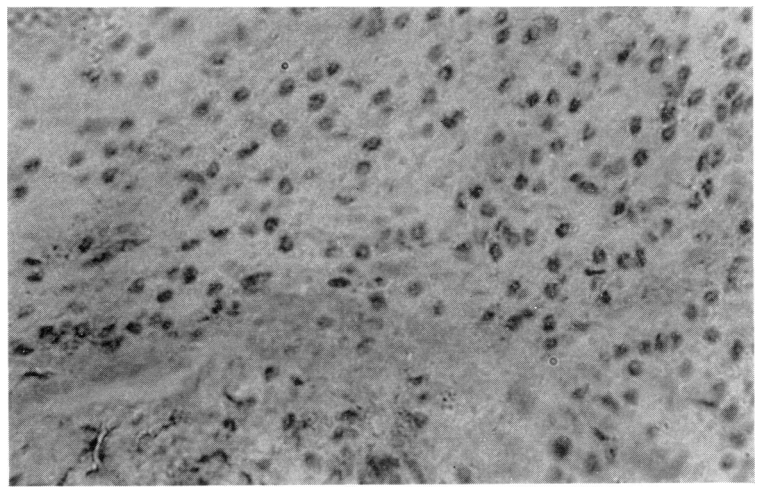

FIG. 7. Footpad reaction in a recipient of mycobacterium antiserum 3 hours after i.d. challenge with MCF. Note heavy polymorphonuclear cell infiltration. Haematoxylin and eosin stain. (× 480.)

The mean values of footpad swelling at 24 hours in each of the three groups of immune cell recipients showed significantly higher ($P<0.005$) results than that of antiserum recipients.

The immediate or delayed character of the footpad reactions was further analysed by histology. Taking the mycobacterium system as a representative sample, histological sections were prepared from the injected, amputated footpads of serum and cell recipients, at 3 and 24 hours respectively. Serum recipients at 3 hours after antigen injection reacted with a characteristic infiltration of polymorphonuclear reaction, while recipients of immune cells displayed a typical mononuclear infiltration at 24 hours (Figs 7 and 8).

Furthermore, several of the recipients of mycobacterium immune cells were skin tested immediately after the transfer. Footpad swelling at 17 hours was 0·48 mm and at 24 hours 0·32 mm. Histology of these pads also showed a typical mononuclear infiltration.

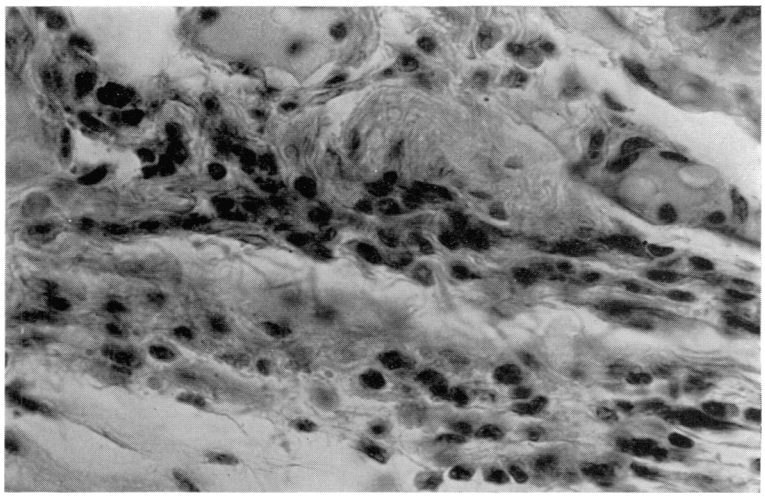

FIG. 8. Footpad reaction in a recipient of mycobacterium-immune lymphoid cells 24 hours after intradermal challenge with MCF. Note mononuclear cell infiltration. Haematoxylin and eosin stain. (× 720.)

GRANULOMA FORMATION AROUND BARE PARTICLES OR PARTICLES COATED WITH A HETEROLOGOUS ANTIGEN DURING CONCOMITANT, SPECIFIC, SYSTEMIC ANTIGENIC STIMULUS

In the foregoing experiments it was established that granuloma formation around antigen-coated bentonite particles is a specific, cell-mediated reaction. Since the infectious type bentonite granuloma is composed mainly of macrophages and their derivatives, it was hypothesized, in keeping with recent concepts, that recruitment and immobilization of these cells around the antigenic foci would be enhanced following a systemic antigenic stimulus, resulting in lymphocyte-macrophage interaction.

In the experiments described below, this concept was tested by specifically stimulating sensitized animals and observing granuloma formation around bare particles or those coated with a heterologous antigen. In the first series of experiments, mice with schistosome infections of 7 weeks duration were used. This time period was chosen because while the animals do evince delayed hypersensitivity, no eggs are yet to be found in the lungs. Results presented in Table 4 show that mice which had been repeatedly injected intra-

TABLE 4

GRANULOMA FORMATION AROUND UNCOATED BENTONITE PARTICLES OR THOSE COATED WITH HETEROLOGOUS ANTIGENS ELICITED BY I.V. INJECTIONS OF SOLUBLE EGG ANTIGENS (SEA) IN SCHISTOSOME-SENSITIZED MICE

	Antigenic stimulus	Bentonite particles coated with:		
		Uncoated	MCF	HCF
		Mean granuloma diameter, $\mu m \pm SE$		
Schistosoma infection	SEA	99±2 (7/125)*	97±5 (7/94)	104±4 (6/98)
	PBS	79±2 (7/113)	68±3 (5/82)	82±3 (6/85)
SEA+FCA, s.c.	SEA	123±5 (5/ 55)	—†	100±4 (6/74)
	PBS	86±2 (5/ 81)	—	74±3 (6/82)

* Number mice/number lesions read. † Not done as FCA contains Mycobacteria.

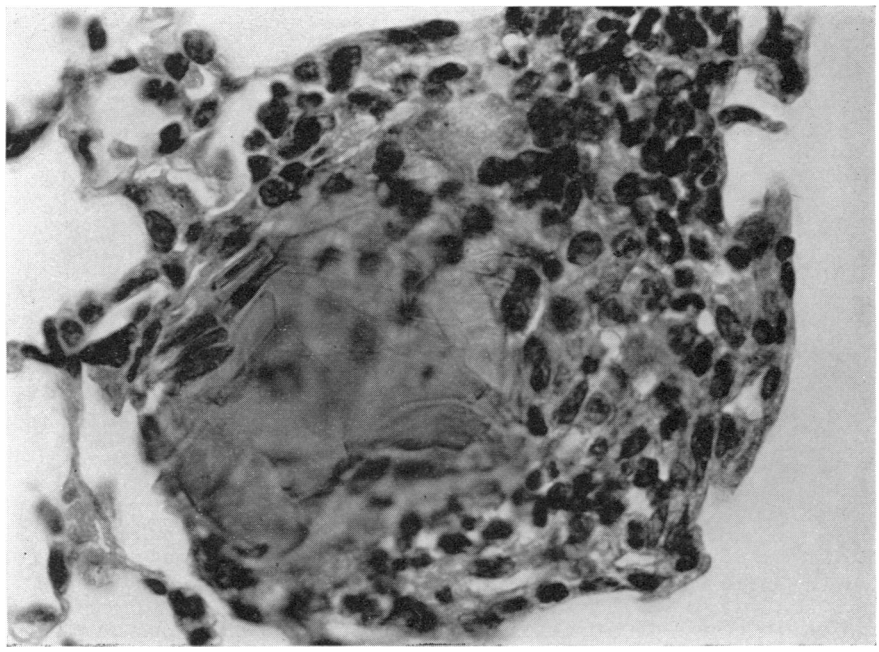

FIG. 9. Granuloma formation at 4 days around a bare bentonite particle in the lung of a schistosome-infected mouse i.v. injected with SEA. Haematoxylin and eosin stain. (× 720.)

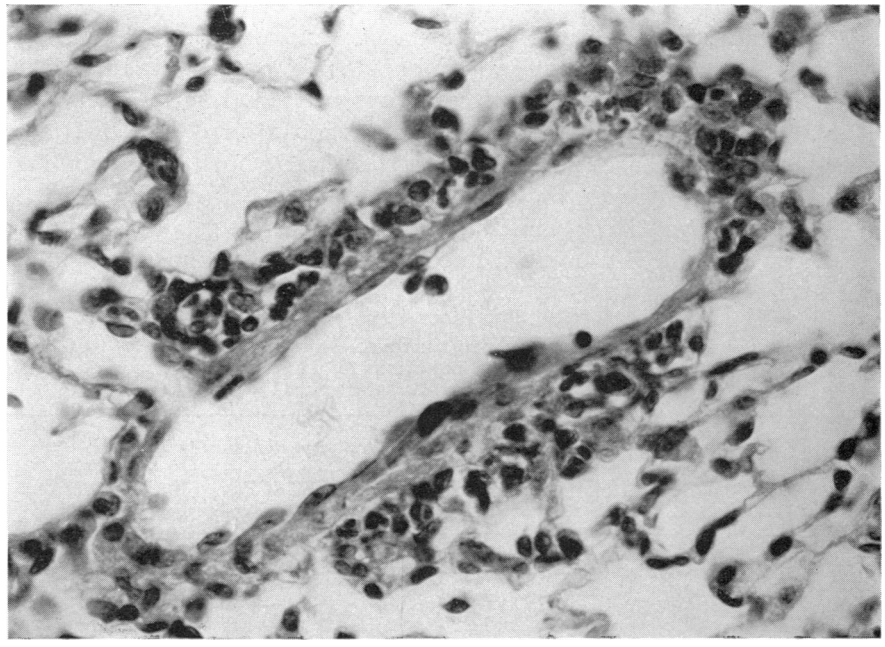

FIG. 10. Perivascular infiltration of mononuclear cells in the lungs of a schistosome-infected mouse following i.v. injections with SEA. Haematoxylin and eosin stain. (× 600.)

venously with the specific antigen (SEA) during the period of granuloma formation, reacted with significantly larger granulomas than control, buffer-injected animals. It made no difference whether bare particles or particles coated with heterologous antigen were injected into the lungs of the animals; once a nidus was provided, cells gathered around it (Fig. 9). Stained sections of lungs thus stimulated, showed perivascular cuffing by mononuclear cells (Fig. 10) which were also dispersed in the alveoli. Eosinophils were likewise scattered in the lungs. These same cells made up the granulomatous lesions around the bentonite particles. Similar observations were made in three to four mice infected with tubercle bacilli and injected with SEA-coated particles during continuous stimulation with MCF (experimental—89 ± 3 μm; saline control—66 ± 2 μm). Histoplasma-infected mice challenged with SEA-coated particles and stimulated with HCF showed similar results (experimental—92 ± 5 μm; saline control—72 ± 6 μm).

While differences in granuloma size between specifically stimulated and control mice were statistically significant, even lungs of buffer-injected mice showed some cell mobilization around blood vessels and in tissues. This excited state was attributed to the endogenous antigenic simulus of the infected animals. To work in a 'cleaner' system, the same experiments were repeated with antigen-adjuvant sensitized mice. Results were essentially the same as in the infected mice, but with somewhat greater differences in granuloma size between stimulated and control animals.

DISCUSSION

The relationship between granulomatous inflammation and delayed hypersensitivity has been debated for many years. Ever since the classic experiments of Koch, delayed hypersensitivity has been linked to and identified by the cutaneous reactivity of sensitized individuals to tuberculoprotein. While it was recognized that the mycobacterium evokes a tuberculous lesion in the tissues of infected man or animals, its relation to the state of cutaneous reactivity was uncertain. An obvious difficulty in the understanding of the granulomatous inflammation was the lack of differentiation between the cellular response appearing around a foreign object (foreign-body granuloma) and that of the hypersensitive (allergic, infectious) granuloma which is generated in response to infectious agents. That two different cell components of the tubercle bacillus (tuberculoprotein and Wax D) could elicit dermal hypersensitivity or foreign-body granulomatous reactivity, respectively, only added to the difficulties of interpretation. Thus Rich concluded in 1951 that tubercle and epithelioid cell formation is primarily a response to the bacillary lipid and that hypersensitivity is not essential for tubercle formation. Several years later Lurie (1964) stated that the tuberculous granuloma caused by dead tubercle bacilli is the result of the irritating effect of the lipid constituents. A similar opinion was expressed by Raffel in a review published in 1971. An additional, and as yet not quite explained phenomenon is the dissociation between dermal and granulomatous hypersensitivity which has been produced by desensitization in experimental animals with tuberculosis, or which occasionally occurs in diseases such as sarccoidosis, Hodgkin's, etc. The prevailing uncertainty concerning the allergic granulomas was expressed in 1966 by Uhr who, in a major review of delayed hypersensitivity, concluded 'the relationship between delayed hypersensitivity and the formation of granulomata is not known,' and in two recent reviews in which the authors spoke of insufficient evidence for the inclusion of granuloma in the delayed responses (Epstein, 1967) and the 'close, but mysterious association of granulomas with delayed hypersensitivity' (Spector, 1969).

In previous publications from our laboratory a novel experimental approach was suggested for the study of granulomatous hypersensitivity. Using uncoated bentonite particles or particles coated with soluble antigens of schistosome eggs (Boros and Warren, 1970), or mycobacteria (Boros and Warren, 1971), foreign-body or hypersensitivity type granulomas could be differentially evoked in the lungs of normal or sensitized mice. Differences between the two granuloma types could be demonstrated with respect to size and cellular composition of the lesions. In the present experiments, these preliminary observations were further extended and investigated in greater detail. Substituting antigen-coated particles for the actual granuloma-evoking organisms, granuloma formation could be elicited in sensitized mice, thus simulating the events occurring during infection with schistosomes, tubercle bacilli or histoplasma.

The bentonite granuloma model system is a uniquely versatile one. Not only can it be utilized for the study of the hypersensitivity granuloma and the foreign body granuloma, but the latter can even be transformed into the former under certain circumstances, the determining factor being the presence or absence of cell-mediated immunity. The features of the hypersensitivy granuloma elicited by particles coated with antigen in homologously sensitized animals that conform to the criteria for delayed hypersensitivity are: (1) Hypersensitivity-type granulomas seem to be associated with the state of delayed hypersensitivity (as manifested by delayed footpad swelling). (2) Granuloma formation is immunologically specific. (3) It is transferrable by immune lymphoid cells, but not by antiserum. (4) Hypersensitivity-type granulomas are composed of lymphocytes, macrophages, epithelioid cells, and occasional oesinophils.

The foreign body reaction around uncoated or heterologously coated bentonite particles in unsensitized or sensitized animals differs from the hypersensitivity type reaction in the following respects: (1) There is no anamnestic reaction following previous exposures, and granuloma formation is not immunologically specific. (2) The granuloma is smaller and cellular composition consists mainly of histiocytes. (3) There is a possible activation of chemical mediators of inflammation as shown in a previous study using plastic beads (Kellermeyer and Warren, 1970).

The foreign body type granuloma can, however, be converted into the hypersensitivity type by the systemic injection of homologous antigen into specifically sensitized animals. Inflammation occurs around these sessile particles in the tissues which has all the cellular characteristics of the hypersensitivity granuloma.

Taken together, these observations point to the sensitized lymphocyte as the specific, key element in the generation of the hypersensitivity bentonite granuloma and strongly indicate that these reactions are expressions of cell-mediated immunity. This is consonant with earlier observations in which granulomas formed around schistosome eggs could be suppressed by immunosuppressive measures primarily functional in cell-mediated immunity (Domingo and Warren, 1967; 1968). It is recognized that in this kind of immune reaction two cell types participate: the sensitized lymphocyte and the bone marrow-derived and blood-borne monocyte. Interaction between these cells is carried out by soluble mediators (Bloom, 1971) released by lymphocytes after specific contact with an antigen. These soluble mediators are capable of mobilizing and activating not only the bone marrow-derived monocytes, but possibly also the eosinophils as was shown recently (Basten and Beeson, 1970; Cohen and Ward, 1971).

While the present experiments do not furnish evidence for the participation of soluble mediators in the formation of the bentonite granuloma, it is attractive to assign a tentative

role to mediators such as migration inhibitory factor (Bloom and Bennett, 1966; David, 1966), chemotactic factor (Ward, Remold, and David, 1969), etc. as logical intermediaries in the generation of the granuloma. In this light, generation of hypersensitivity-type granulomas around bare particles during the specific systemic stimulation of sensitized mice can be better explained. Yoshida and associates (1969) demonstrated a significant increase in the number of circulating blood monocytes in systemically stimulated sensitized animals which was attributed to a systemic release of migration inhibitory factor (MIF). Salvin and Youngner (1972) in a preliminary communication reported that a systemic stimulus of old tuberculin given to bacille Calmette-Guérin (BCG)-infected hypersensitive mice elicited the appearance of MIF in the sera of animals. In our experiments, concomitant systemic administration of antigen in specifically sensitized animals resulted in large granulomas and evoked the appearance of numerous macrophages and eosinophils in the tissues and around the blood vessels of the lung. Thus, it seems that granulomas can be generated not only around a specific antigenic focus, but also around any nidus which happens to be in the way of the 'angry' migrating macrophages. Thus, in granuloma formation a cooperation between lymphocytes and macrophages is evident which seems to be analogous to that described by Mackaness (1969; 1970) for non-specific anti-microbial immunity. The non-specific formation of hypersensitivity granulomas might serve as a basis for the understanding of granulomatous diseases of unknown etiology. Experiments presently underway using MIF-coated bentonite particles may provide direct proof for the participation of this or other factors in granuloma formation.

By and large, in the mycobacterium and histoplasma system granuloma formation around coated particles correlated well with dermal reactivity as manifested by delayed footpad swelling. Essentially no delayed footpad swelling was observed in schistosome-infected animals, but egg sensitized or SEA-adjuvant sensitized animals did display good reactions. Similarly, recipients of lymphoid cells transferred from schistosome-infected mice displayed only weak dermal, but strong granulomatous reactions. This divergence between the granulomatous and dermal reactivity in schistosomiasis is as yet unexplained.

Since the soluble antigens employed in all three models are products of the respective infectious organisms, it might be assumed that the same substances participate also in the initiation of the granulomatous process in the diseased state. The antigens employed were a relatively crude mixture of proteinaceous and carbohydrate-containing materials (Boros and Warren, 1970; Daniel and Ferguson, 1970; Walter and Price, 1968). However, the same model could be proposed for the investigation of purified protein, carbohydrate and lipid fractions of different organisms in the analyses of the granulomatous response. The first step in this direction has been taken, employing lysophosphatide-coated bentonite particles which, when injected, were shown to evoke a non-specific granulomatous response (Smith, Lucia, Doughty and von Lichtenberg, 1971). In our laboratory, experiments have been undertaken with bentonite particles coated with purified protein- and carbohydrate-containing fractions of the mycobacterial culture filtrate.

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