*DELAYED HYPERSENSITIVITY IN VITRO: ITS MEDIATION BY CELL-FREE SUBSTANCES FORMED BY LYMPHOID CELL-ANTIGEN INTERACTION**

By John R. David†

DEPARTMENT OF MEDICINE, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

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It has long been thought that delayed hypersensitivity is mediated by cells or cell-associated substances. In an attempt to investigate the mechanism of delayed hypersensitivity, *in vitro* studies using the inhibition of cell migration by specific antigen as an assay have been carried out. In this *in vitro* system, it has been shown that peritoneal exudate cells taken from guinea pigs exhibiting delayed hypersensitivity and placed in capillary tubes are inhibited from migration by specific antigen.[1, 2] Moreover, when mixed populations of normal and sensitive cells were prepared, it was observed that if as few as 2.5 per cent of the cells in a population were from a sensitive animal, the whole population (97.5% normal cells) would be inhibited by antigen.[3]

The results of recent experiments suggest that the lymphocyte is the specifically sensitive cell in this system. It was found that lymph node cells (approximately 95% lymphocytes) obtained from sensitive guinea pigs will, when mixed with a population of normal peritoneal exudate cells, cause the whole population to be inhibited by specific antigen.[4] It is of note that such sensitive lymphoid cells, when assayed alone in culture, are not themselves inhibited from migrating by antigen.

Experiments were initiated in an attempt to determine the manner by which these sensitive lymphoid cells achieved their effect. The results, here reported, demonstrate that, following incubation of sensitive lymphoid cells with specific antigen for 24 hr, a nondialyzable substance is detected in the cell-free supernatants which inhibits the migration of normal peritoneal cells.

Materials and Methods.—Sensitization: The antigens used were ovalbumin (Worthington) and o-chlorobenzoyl chloride conjugated to bovine gamma globulin (OCBC-BGG) by the method of Benacerraf and Levine,[5] kindly supplied by Dr. Y. Borel. Guinea pigs of the Hartley strain weighing 500–800 gm were sensitized with the appropriate antigen diluted in saline and emulsified in an equal volume of complete adjuvant (Difco H37Ra). Each animal received a total dose of 100 μg of antigen distributed into the four footpads, 0.1 ml per footpad.

Tissue culture media: The basic tissue culture media used throughout were minimal essential media, Eagle 12-125 (Microbiological Associates, Bethesda, Md.) containing 15% normal guinea pig serum and 85 units of penicillin and 85 μg of streptomycin/ml.

Preparation of lymph node cell suspensions: Twelve to twenty one days after sensitization axillary, inguinal and popliteal lymph nodes were obtained aseptically from guinea pigs which had been anesthetized with ether and exsanguinated by cardiac puncture. The nodes were diced into tissue culture medium, teased gently with mouse-toothed forceps, and the resulting cell suspension was pipetted into centrifuge tubes. The suspension was allowed to stand for 4 min so that the tissue fragments settled by gravity. The supernatant was then removed to a fresh tube. After three such settlings the cell suspensions were essentially free of tissue fragments. The majority of cells were viable as assessed by trypan-blue exclusion, and the preparations contained 90–95% lymphocytes by Wright's stain and phase microscopy. Suspensions were adjusted to a final concentration of 1.8×10^7 cells per ml. Aliquots of these suspensions were made to contain ovalbumin, or OCBC-BGG 100 μg/ml. Suspensions not containing antigen were also prepared. In each experiment, suspensions were incubated in specific antigen and an unrelated antigen.

Mackaness type chambers (capacity 0.8 ml or 4 ml) were filled with cell suspensions, sealed, and incubated for 24 hr at 37°C. The suspensions were recollected, spun at 2300 rpm for 30 min, and the supernatants passed through sterilizing Millipore filters 0.4 μ to ensure that they were cell-free. The pH was adjusted to 7.4 with 5% CO_2 and air as required.

Dialysis: The supernatants collected as described above were divided into two aliquots, and 4.5 ml were dialyzed against 100 ml of tissue culture media at 4°C under 5% CO_2 to maintain constant pH. An equal volume was kept cold but not dialyzed. After 24 hr the supernatants were centrifuged at 2300 rpm for 30 min, passed through Millipore filters, and used to fill chambers containing normal peritoneal cells in capillary tubes.

Assay for cellular sensitivity: Peritoneal cells from normal guinea pigs were induced by oil and collected as previously described.[2] The cells were washed in balanced salt solution and suspended to 10% vol in tissue culture medium. Capillary tubes were filled with this suspension, sealed with wax, and centrifuged. The tubes were cut, and the portion containing the packed cells was placed in Mackaness type chambers, two tubes per chamber. In each experiment at least two to three chambers were prepared for each media to be tested. The media include supernate from sensitive lymphoid cells incubated with (1) specific antigen, (2) unrelated antigen, (3) without antigen; tissue culture media containing (4) specific antigen, (5) unrelated antigen, and (6) without antigen. The concentration of antigen was 100 μg/ml. It should be noted that where OCBC-BGG was the sensitizing or specific antigen, ovalbumin was used as the unrelated antigen; where ovalbumin was the specific antigen, OCBC-BGG was the unrelated. Thus, in every experiment lymphoid cells were incubated both in specific and unrelated antigen. Chambers were incubated for 24 hr, and the area of migration was measured by planimetry as described previously.[2] In calculating the data from these experiments, the following formula was used:

$$\frac{\text{Average area of migration in supernatant with antigen}}{\text{Average area of migration in media with the same antigen}} \times 100 = \% \text{ migration.}$$

Results.—Cell-free supernatants from sensitive lymph node cells incubated 24 hr with specific antigen produced inhibition of migration of normal peritoneal exudate cells. In contrast, supernatants from the same cells incubated with an unrelated antigen, or incubated without antigen, had no effect.

In 14 experiments the average migration of normal peritoneal exudate cells in supernatants prepared with specific antigen was 52 per cent as compared to migration in control media containing the same antigen. The average migration of normal cells in supernatants prepared with unrelated antigen was 97 per cent (see Table 1 and Fig. 1).

In eight additional experiments, the total area of migration was not significantly inhibited; however, there was marked clumping of cells migrating in supernatants prepared with specific antigen which was reminiscent of the effects of antigen on sensitive cells when added after 24 hr of incubation in normal media.[2] This clumping may be a qualitative expression of the same phenomenon as inhibition of migration and a function of the amount of active material present. If the specific supernatant is diluted 1:5 or 1:10, the effect is abolished.

TABLE 1

MIGRATION OF NORMAL PERITONEAL EXUDATE CELLS IN SUPERNATANTS FROM SENSITIVE LYMPHOID CELLS

Per Cent Migration	
Specific* antigen	Unrelated* antigen
19†	89
20	82
40	122
43	79
50	81
53	110
53	81
54	99
54	97
65	117
65	—
72	—
73	102
73	100
Average	
52	97

* Antigen incubated with sensitive lymphoid cells.
† All figures represent average migration in supernatant compared to migration in media with same antigen.

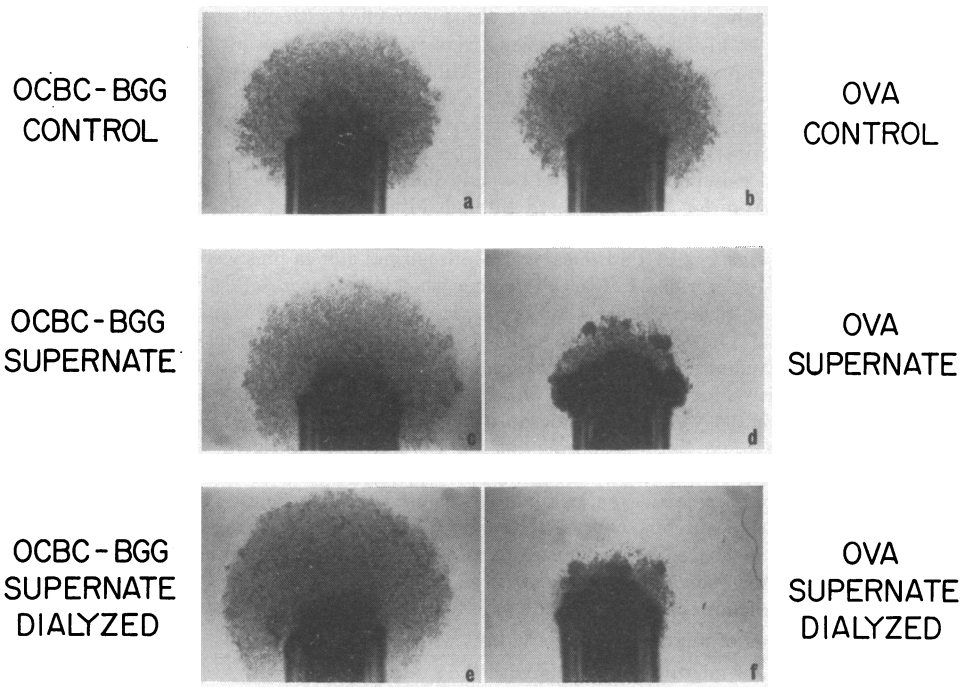

FIG. 1.—Effect of supernatants from ovalbumin-sensitive lymph node cells incubated with specific and unrelated antigens on the migration of normal peritoneal exudate cells. (*a*) Normal peritoneal cells in media containing OCBC-BGG. (*b*) Normal cells migrating in media containing ovalbumin. (*c*) Normal cells migrating in supernatant from ovalbumin-sensitive lymph node cells incubated with OCBC-BGG. (*d*) Normal cells inhibited by supernatants from ovalbumin-sensitive lymph node cells incubated with ovalbumin. (*e*) Same as (*c*) except that supernatant was dialyzed for 24 hr. (*f*) Same as (*d*) except that supernatant was dialyzed for 24 hr. Peritoneal cells in all photographs came from the same pool.

Addition of antigen to supernatants after 24 hr of incubation: The possibility that sensitive lymphoid cells incubated without antigen produced cell-free substances which would subsequently interact with antigen and inhibit the migration of normal peritoneal exudate cells was investigated. Lymphoid cells from guinea pigs with delayed hypersensitivity to OCBC-BGG were incubated without antigen, with OCBC-BGG, and with ovalbumin. After 24 hr the cells were removed by centrifugation and the supernatants passed through Millipore filters. OCBC-BGG was added to half of the supernatant from the group of sensitive cells incubated without antigen. In three experiments, normal peritoneal exudate cells migrated normally in the supernatants to which the specific antigen had been added *after* 24 hr. The average migration was 106 per cent. In contrast, the same cells were inhibited by the supernatant which had been prepared by adding OCBC-BGG *before* the start of incubation, migrating an average of only 51 per cent. Supernatant obtained from cells incubated for 24 hr with ovalbumin, an unrelated antigen, had no effect; the average migration in these chambers was 101 per cent.

Dialysis of supernatants: In four experiments, 4.5 ml of supernatant were dialyzed against 100 ml of tissue culture medium. The results of these experiments are shown on Table 2 and Figure 1. It can be seen that the dialyzed and nondialyzed supernatants prepared with specific antigen were equally effective in

TABLE 2

MIGRATION OF NORMAL PERITONEAL CELLS IN SUPERNATANTS FROM OVA-SENSITIVE LYMPH NODE CELLS

Per Cent Migration			
Ova*		OCBC-BGG*	
Not dialyzed	Dialyzed	Not dialyzed	Dialyzed
54†	46	99	125
53	57	110	135
50	47	81	112
—	63	—	113
Average			
53	53	96	121

* Antigen incubated with ovalbumin-sensitive lymphoid cells.
† All figures represent average migration in supernatant compared to migration in media with same antigen.

inhibiting the migration of normal cells. In contrast, dialysis of supernatants prepared with unrelated antigen consistently allowed the normal peritoneal cells to migrate further than in the nondialyzed media, and no inhibition was observed.

Heat stability: In three experiments, supernatants prepared with specific antigen were divided into two aliquots, and one portion heated to 56°C for 30 min. It was found that normal peritoneal cells were still inhibited by the heated supernatants.

The effect of puromycin on the production of active supernatant: In two experiments, sensitive lymphoid cells were exposed to 5 μg/ml puromycin during 24 hr of incubation with specific antigen; control sensitive cells were incubated in specific antigen alone. After 24 hr the cells were removed by centrifugation and the supernatants dialyzed over 24 hr to remove the puromycin (controls were also dialyzed). The supernatants were assayed for their effect on normal cell migration. The results showed that exposure to puromycin during incubation abolished the ability of supernatants prepared with specific antigen to inhibit normal cell migration; cells migrated normally in these supernatants, averaging 100 per cent. On the other hand, supernatants prepared with specific antigen, but not exposed to puromycin, inhibited the migration of normal cells. The average migration was 54 per cent in one experiment, and marked clumping was observed in the other.

Discussion.—The data presented here indicate that sensitive lymphoid cells during incubation with specific antigen elaborate a soluble substance into the media which inhibits the migration of normal peritoneal exudate cells. This material is not detected in supernatants from lymph node cells incubated with an unrelated antigen, or incubated without antigen, thus suggesting that the production or release of this material is the result of a specific immunologic reaction.

The most attractive interpretation of these findings is that specifically sensitive lymphocytes are stimulated to form inhibitory substances by antigen. Another interpretation could be that the substances are normally produced when the cells are incubated without antigen; but in the presence of antigen, complexes are formed which inhibit the migration of normal cells. Evidence against this interpretation is the finding that the supernatants are ineffective when antigen is added after 24 hr of incubation without antigen. It is not known whether the material stimulated by antigen inhibits the migration of normal peritoneal cells of itself or whether the continued presence of antigen is necessary. All the experiments in these studies have utilized large soluble antigens which were present in the supernatants when added to normal cells.

The results of experiments with puromycin provide additional evidence that the lymphocytes are actively synthesizing material(s) in response to antigen. In this group of experiments, it was found that the presence of puromycin during the incubation of sensitive lymphoid cells with antigen resulted in supernatants which had no effect on normal cells. These results are in agreement with earlier studies where puromycin was shown to prevent the inhibition of migration of sensitive peritoneal cells by antigen and this effect correlated with inhibition of protein synthesis by puromycin.[6]

In the course of the present experiments, it was observed that the media became less acid in lymphocyte cultures containing puromycin than in control chambers. In addition, it was found that more nonviable cells were present in chambers incubated with puromycin than in control chambers. Therefore, the puromycin may be producing its effect by inhibiting protein synthesis, or by a lethal effect on the lymphoid cells secondary to this inhibition. It should be noted that cells destroyed in this manner did not release substances into the media which inhibited the migration of normal cells. This result provides further evidence against the existence of preformed inhibitory materials.

Previous attempts to detect cell-free substances which would inhibit the migration of normal cells from sensitive peritoneal exudate cells (approximately 20% lymphocytes) have been unsuccessful.[3] These experiments were repeated with sensitive lymph node cells (approximately 95% lymphocytes). Chambers were prepared to contain one capillary filled with normal peritoneal cells adjacent to two capillaries with sensitive lymph node cells. When antigen was present in these chambers, a marked clumping of the normal cells was observed (the sensitive lymphoid cells migrated normally). A possible explanation for failures to produce this effect with sensitive peritoneal cells may be that the material produced by the lymphocytes in the sensitive peritoneal populations is immediately taken up by the surrounding macrophages and little diffuses out into the media. In contrast, the material appears readily to diffuse away from populations containing a majority of lymphocytes.

Recently, an abstract by Bloom and Bennett has appeared describing experiments using lymphocytes from peritoneal exudates obtained from animals sensitive to PPD. Supernatants of these cells incubated with PPD inhibited the migration of normal peritoneal cells, whereas sensitive lymphocytes incubated without antigen or normal cells incubated with PPD had no effect.[7]

The data available suggest an explanation of the phenomenon of inhibition of peritoneal cell migration by specific antigen. The first event may be the reaction of specifically sensitive lymphocytes in the population with antigen. This interaction results in the production of substances that, either alone or in combination with antigen, affect the remaining nonsensitive cells, presumably macrophages, to inhibit the migration of the whole population.

It is of interest that mixed populations containing 20 per cent sensitive peritoneal cells and 80 per cent *normal peritoneal* cells are inhibited by antigen; in contrast, other populations containing 20 per cent of the same sensitive peritoneal cells and 80 per cent *normal spleen* cells are not inhibited by specific antigen.[4] Furthermore, sensitive lymphoid cells themselves are not inhibited by specific antigen. These findings suggest that two cell types are important in these reactions, sensitive

lymphocytes and cells from the peritoneal exudate, presumably macrophages, which need not be sensitive.

Further studies on the supernatants prepared with specific antigen indicate that the inhibitory material is nondialyzable and stable after incubation at 56°C for 30 min.

The detection of a material with these biological properties raises interesting questions as to its nature, whether the continued presence of antigen is required for its effect, its relation to known immunoglobulins, and its relevance to *in vivo* events in delayed hypersensitivity.

Summary.—The data presented indicate that sensitive lymphoid cells (95% lymphocytes), during incubation with specific antigen, elaborate a soluble substance into the media which inhibits the migration of normal peritoneal exudate cells. The material is not found in supernatants from sensitive lymph node cells incubated with an unrelated antigen, or incubated without antigen. When the specific antigen is added to supernatants from sensitive cells incubated for 24 hr without antigen, the supernatants have no effect on normal cells. The production of this material is inhibited by puromycin. These experiments suggest that specific antigen stimulates sensitive lymphoid cells to produce soluble materials which inhibit the *in vitro* migration of normal peritoneal exudate cells. The inhibitory substance is nondialyzable, and active after heating at 56°C for 30 min.

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† Career Scientist of the Health Research Council of the City of New York. Present address: Robert B. Brigham Hospital, Boston, Massachusetts.

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