Table 1. Pattern of plug formation by *aa* and $A^{y}a$ males.

Genotype		Plugs on days after placement with the male (No.)				
Male	Female (and number)	Day 1	Day 2	Day 3 (and percentage)	Day 4	and later Day 5
aa	*aa*(51)	11	13	19(37%)	4	4
aa	$A^{y}a$(134)	28	27	50(37%)	22	7
$A^{y}a$	*aa*(125)	26	25	28(22%)	18	28
$A^{y}a$	$A^{y}a$(52)	12	15	11(21%)	7	7

Table 2. Statistical analysis of number of females remaining unfertilized at the end of each day.

Item	Day 2	End of day 3	Day 4
Total χ^2 (df:3)	3.20	10.86*	9.18‡
aa ♀♀	1.25	0.56	0.40
$A^{y}a$ ♀♀	1.18	.52	.37
Between ♀♀ (df:2)	2.43	1.08	.77
Within ♀♀ (df:1)	0.77	9.78†	8.41*
aa ♂♂	.87	4.47	4.38
$A^{y}a$ ♂♂	.91	4.68	4.59
Between ♂♂ (df:2)	1.78	9.15*	8.97*
Within ♂♂ (df:1)	1.42	1.71	0.21

* P = .01 to .02. † $P < .01$. ‡ P = .02 to .05.

10 days the females were checked daily for the presence of vaginal plugs. Conditions of the experiment were kept constant from one series to the next, and data from several consecutive runs were pooled for analysis.

The data (Table 1) were analyzed by means of a χ^2 test on the number of females remaining unfertilized in each category each day; the expected numbers were calculated from the initial group and from the number remaining unfertilized at the end of each day; presence of the Whitten effect would cause significant departure from randomness of the number of females fertilized on the 3rd day. Partitioning of χ^2 (Table 2) showed deviations from randomness at the .01 level "within females" and "between males" on days 3 and 4; there were no significant deviations "between females" or "within males." In matings of *aa* males with both genotypes of females, a higher proportion were fertilized on day 3 and a smaller proportion on day 4 than in matings of $A^{y}a$ males with either *aa* or $A^{y}a$ females. Thus estrous synchrony was induced by *aa* males but not by $A^{y}a$ males and was not influenced by the genotype of the females.

Among females that mated during the experiment, 94 percent of those placed with *aa* males mated during the first 4 days, while only 80 percent of those placed with $A^{y}a$ males mated during this period ($\chi^2 = 15.6$, $P < .001$).

The lethal yellow (A^{y}) gene in heterozygous state affects hair pigmentation, fat and cholesterol metabolism, development of spontaneous and induced tumors, and normal growth (*4*). In the YS/ChWf strain, reproductive function is also affected by this gene (*3*). Because of higher embryonic mortality, litter size is smaller in $A^{y}a$ females mated with *aa* males than in the reciprocal matings. Yellow females mated to yellow males produce, on the average, only half as many litters as (1.3 : 2.5) and cease production at an earlier age (18.0 : 23.7 weeks of age at birth of last litter) than when mated to nonyellow males.

These data indicate that, in the YS/ChWf strain, yellow ($A^{y}a$) males do not elicit synchronous estrus in females previously kept in groups, while nonyellow (*aa*) males do so regularly. The genotype of the female (*aa* or $A^{y}a$) does not significantly affect this phenomenon. The Whitten effect is believed to be caused by the olfactory stimuli provided by a mature male (*1*). Possibly $A^{y}a$ males differ significantly from *aa* males in the production of the odoriferous substance concerned and, consequently, do not provide sufficient olfactory stimulation to influence the estrous cycle to a measurable extent.

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Mechanism of a Reaction in Vitro Associated with Delayed-Type Hypersensitivity

Abstract. *The cell type responsible for inhibition by antigen of migration in vitro of peritoneal exudate cells obtained from tuberculin-hypersensitive guinea pigs was studied. Exudate populations were separated into component cell types, the lymphocyte and the macrophage. Peritoneal lymphocytes from sensitive donors were the immunologically active cells in this system, the macrophages being merely indicator cells which migrate. Sensitized peritoneal lymphocyte populations, upon interaction with specific antigen in vitro, elaborated into the medium a soluble material capable of inhibiting migration of normal exudate cells.*

The nature of delayed-type hypersensitivity remains a problem for two reasons. The cell type or types which effect the delayed-type reactions are not established with certainty (*1*), and the biochemical basis of the response is almost completely unknown. In order to approach these problems under defined conditions, a great deal of work has been directed towards developing methods for studying delayed-type hypersensitivity in vitro (*2*). The discovery (*3*) that migration of cells from splenic explants from hypersensitive guinea pigs was specifically inhibited by antigen has served as the basis for a quantitative assay (*4*) which is antigen-specific, reproducible, and apparently independent of the antibody response. In this method, peritoneal exudate cells are allowed to migrate from capillary tubes onto cover slips in small culture chambers, and the area of cell migration is measured. The migration of exudate cells obtained from hypersensitive guinea pigs is markedly inhibited by the presence in the medium of specific antigen. This inhibition of migration seems characteristic of cells from animals with delayed-type hypersensitivity, since exudate cells obtained from nonhypersensitive animals immunized to produce only circulating antibody are not inhibited by antigen (*4, 5*). In addition, the results obtained from this assay in vitro correlate well in

other respects with observations of delayed-type hypersensitivity in vivo, in that killed cells, cell extracts, or living cells whose protein synthesizing capacity has been inhibited fail to effect the reaction (*5, 6*).

In this system a very small number of exudate cells, obtained from hypersensitive animals, admixed with exudate cells from normal animals was sufficient, in the presence of antigen, to produce inhibition of migration of the total cell population (*5*). This observation led us to question which cell type was immunologically active in view of the facts that (i) the peritoneal exudate is the most active cell source in passively transferring delayed-type hypersensitivity in vivo (*6*), and (ii) peritoneal populations contain both macrophages (~ 75 percent) and lymphocytes (~ 15 percent). Both cell types have been implicated as mediators of delayed-type hypersensitivity (*1*). We, therefore, devised means for separating and purifying both peritoneal lymphocytes and macrophages from hypersensitive guinea pigs and determined which was immunologically active in the system in vitro.

Hartley guinea pigs (600-800g) were sensitized to tuberculin by the injection of Freund's adjuvant (3.3 mg of H37Ra mycobacteria per milliliter, Difco) as follows: 0.1 ml into each foot pad and 0.6 ml into nuchal muscles. After an interval of at least 3 weeks, these animals were tested intradermally with 10 μg of tuberculin purified protein derivative (PPD), and those exhibiting delayed reactions of 15 mm or more were used as cell donors (*7*). For purposes of separating cell types, peritoneal exudates were induced by intraperitoneal injection of sterile 2.5 percent starch gel in saline, 72 hours before harvesting the cells; in other experiments, exudates were produced either with starch or by injection of 25 ml of sterile paraffin oil 48 hours before obtaining cells. The general method for separating peritoneal macrophages from lymphocytes was as follows. Peritoneal exudate cells were aseptically collected in cold heparinized Hanks' solution; they were then washed and suspended in Eagle's minimal essential medium containing 15 percent normal guinea pig serum (NGPS). The cells were then cultured for 2 hours at 37°C in 10 percent CO_2 in air (16×10^6 cells in each 100-mm glass or plastic petri dish), during which time most of the macrophages attached and spread on the culture surface. The nonadherent cells, mostly lymphocytes, were suspended by gently shaking the plates, and they were removed with the suspending medium. To obtain isolated lymphocytes, cells which did not become attached at the first plating were replated in fresh medium for a second incubation period. After three to four cycles of incubation and resuspension, the remaining nonadherent cells consisted of more than 90 percent lymphocytes. For isolation of macrophages, the original plates from which free lymphocytes had been removed were incubated again in fresh medium for 4 to 24 hours, and the nonattached cells were removed by moderately vigorous shaking of the plates. The attached cells were then released from the glass surface by treatment for 6 to 12 minutes with ethylenediaminetetraacetate (1:5000, Grand Island Biologicals) and were found to be 99.4 to 99.8 percent macrophages.

To test the activity of the purified macrophage populations obtained from hypersensitive donors, macrophages (2.5×10^6/0.1 ml) suspended in medium 199 containing 15 percent serum (NGPS-199) were drawn into capillary tubes and centrifuged at 125*g* for 2 minutes. Tubes were cut at the cell-medium interface, and duplicates were placed in one chamber (*8*) filled with NGPS-199 alone; another set was placed in a second chamber filled with medium containing PPD (20 μg/ml). The activity of purified lymphocytes from the same exudates was also tested. Since lymphocytes alone do not migrate from the tubes, the activity of this cell type was tested by adding the sensitized lymphocyte populations to exudate cells or to purified macrophage populations from unsensitized (normal) guinea pigs. Controls consisted of the same cell mixtures in chambers without PPD, and the normal exudate cells in chambers with PPD. Areas of cell migration were determined from projections of photomicrographs of the chambers taken at 24 and 48 hours.

The degree of inhibition in three experiments is shown in Table 1. Migration was not inhibited in the control chambers, by definition. Histologic examination of cells from migration chambers revealed that the macrophages remained viable even though their migration was inhibited. It is therefore apparent that, in this system, migration of the peritoneal macrophage is not itself directly affected by antigen, but merely serves as an indicator of the immunological activity of the sensitized peritoneal lymphocyte.

Table 1. Percentage inhibition (mean ± S.E.) by tuberculin (PPD) of migration in vitro of various peritoneal cell populations prepared from tuberculin-hypersensitive guinea pigs. The percentage of inhibition of migration is equal to

$$100 - \frac{\text{area of migration in experimental chamber}}{\text{area of migration in control chamber}} \times 100$$

Sensitized lymphocyte addition (%)	Inhibition of migration (%)	
	24 hr	48 hr
*Exudates (from sensitized animals)**		
None	59 ± 8.0	45 ± 9.8
*Macrophages (from sensitized animals)**		
None	0 ± 4.0	
Exudates (from normal animals)†*		
10	56 ± 6.9	55 ± 5.1
2	41 ± 7.4	47 ± 5.0
0.6	22 ± 11.9	27 ± 6.9
0.3	0 ± 6.8	2 ± 2.9

* Control: migration of same cells in medium not containing PPD. † Additional control: migration of normal exudate cells in medium containing PPD.

That the presence of a very few sensitized lymphocytes resulted in migration inhibition of normal macrophages suggested that this specific inhibition was mediated indirectly through the elaboration by the lymphocytes of a soluble material. To investigate this possibility, portions of purified peritoneal-lymphocyte populations from tuberculin-hypersensitive donors were cultured in the presence or absence of PPD. Twenty hours later, the cells were removed by gentle centrifugation, and the cell-free supernatants of the cultures were further clarified by centrifugation at 1000*g* for 15 minutes. Peritoneal-exudate cells obtained from normal donors were resuspended in portions of these supernatants, centrifuged in the capillary tubes as described, and placed in chambers which were then filled with the remainder of the same supernatant fluids. Supernatants from sensitized lymphocytes incubated in the presence of PPD produced marked inhibition of migration of normal exudate cells (Table 2, Fig. 1). On the other hand, supernatants from a variety of control preparations invariably failed to cause migration inhibition. These included: (i) supernatants from normal lymphocytes incubated with or without PPD (not

Table 2. Effects of culture supernatants from sensitized lymphocytes on the migration of normal peritoneal exudate cells (means ± S.E.).

Lymphocytes cultured in*	Inhibition of migration (%)	
	24 hr	48 hr
Medium alone	0	0
Medium alone; PPD added after removal of cells	15 ± 4.6	7 ± 6.0
Medium with PPD	62 ± 3.1	51 ± 5.6

* Purified peritoneal lymphocytes (7 to 10 × 10^6) obtained from tuberculin-hypersensitive guinea pigs were cultured in 1.5 ml 15-percent NGPS-199 for 20 hours in Leighton tubes with or without PPD (25 μg/ml).

shown in table); (ii) supernatants from sensitized lymphocytes incubated either without PPD or with a heterologous antigen (such as, coccidiodin, *9*); and (iii) supernatants in which sensitized lymphocytes had been incubated overnight without PPD, but to which PPD had been added after removal of the lymphocytes. In addition, supernatants of peritoneal-lymphocyte preparations from guinea pigs which had been immunized to produce high titers of circulating antibody, but no delayed-type hypersensitivity (*10*), to bovine serum albumin (BSA), PPD, or ovalbumin (OA) were not inhibitory, in the presence or absence of antigen, to the migration of normal exudate cells. These controls indicate that the inhibitory substance is elaborated only by cells from guinea pigs with delayed-type hypersensitivity, and not by cells from normal animals or from guinea pigs producing circulating antibody. Furthermore, sensitized lymphocytes only produce the inhibitory material when stimulated by specific antigen.

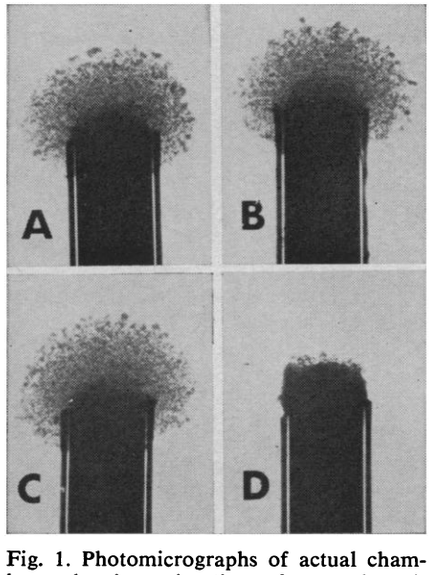

Fig. 1. Photomicrographs of actual chambers, showing migration of normal peritoneal exudate cells in supernatants from 20-hour cultures of purified peritoneal lymphocytes obtained from tuberculin-hypersensitive guinea pigs. Chambers contained supernatants of cells cultured in: (*A*) medium alone; (*B*) medium containing coccidiodin (25 μg/ml); (*C*) medium alone, PPD (25 μg/ml) added after removal of the lymphocytes at 20 hours; (*D*) medium containing PPD (25 μg/ml). (× 8)

The nature of the inhibitory material in the supernatant is unknown, but it may be a protein. David (*11*) demonstrated that antigen-induced inhibition of migration of sensitized whole exudate populations did not occur when puromycin, an inhibitor of protein synthesis, was added to the medium. In confirmation of this, sensitized lymphocytes, whose synthesis of RNA and protein had been blocked by prior treatment for 30 minutes with mitomycin C at 50 μg/ml (*6*), no longer yielded inhibitory material in the supernatant. Moreover the migration-inhibiting factor is not dialyzable.

Also, under certain conditions, migration of normal macrophages could be inhibited simply by antigen and specific antiserum. Immune serums were collected from guinea pigs immunized with BSA or OA to produce circulating antibody as before (*10*), and, in addition, antiserums to PPD were obtained from animals stimulated with Freund's adjuvant, that were repeatedly tested with PPD until strong Arthus reactions were obtained. When normal exudate cells were suspended and allowed to migrate in medium 199 containing 15 percent of the immune serum and respective antigen (BSA and OA, 60 μg/ml and 125 μg/ml; PPD, 25 μg/ml), clear inhibition of migration resulted.

Our experiments indicate that inhibition of migration of macrophages in vitro is mediated by the sensitized lymphocyte through the elaboration of a soluble material produced only in the presence of specific antigen. At least three possibilities can be proposed to explain this reaction in vitro, none in contradiction to what is known about delayed hypersensitivity in vivo. One possibility is that the reaction is produced by an ordinary circulating antibody and is not unique to delayed-type hypersensitivity, even though inhibition of migration has not been found with cells or supernatants derived from antibody-producing guinea pigs. A second mechanism would be that the interaction of sensitized lymphocytes and specific antigen causes the release by the cells of some pharmacologic agent which suppresses migration of macrophages without killing them. A third mechanism is that the sensitized lymphocyte, only upon contact with specific antigen, may be induced to elaborate an antibody-like substance which becomes adsorbed to target cells in the form of antigen-antibody complexes and thereby produces the effect. If this last hypothesis were true, this in vitro system would provide new means of elucidating the mechanism of delayed-type hypersensitivity.

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8. The chambers were a modification of that described by G. B. Mackaness [*J. Pathol. Bacteriol.* **64**, 429 (1952)]. Lucite sheets (5 by 30 by 75 mm) were drilled with one or two holes (13 to 17 mm). Glass cover slips were sealed over the holes on one side with paraffin. Capillary tubes (15-mm diameter) were fixed onto the cover slips with silicone (Dow high-vacuum silicone grease), and the chamber was closed with a second cover slip and paraffin. Medium (0.8 to 1.2 ml) was placed in the chambers through a previously drilled 2-mm access hole.
9. By Cutter Laboratories, Berkeley, Calif.
10. Guinea pigs were injected with 10 mg of BSA or OA, adsorbed to 10 mg of alumina gel or to 0.5 mg of PPD on 5 mg of alumina, in multiple intradermal sites on five occasions over a 10-day period. Serums were obtained 24 days after the last injection of OA. In the case of BSA, animals were given booster injections, 28 days after the last intradermal injection, of 5 mg of BSA in saline, and were bled 7 days later. Titers of hemagglutinating antibody were, for antiserum to OA, 1:5280, and for antiserum to BSA, 1:10,560, with antigen coupled to formalinized erythrocytes through the bis-diazobenzidine linkage [W. T. Butler, *J. Immunol.* **90**, 663 (1963)].
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