THE IN VITRO DESENSITIZATION OF SENSITIVE CELLS BY TRYPSIN*

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In previous studies, carried out using a method described by George and Vaughan (1), it was demonstrated that peritoneal exudate cells obtained from animals exhibiting delayed hypersensitivity were regularly inhibited from migrating *in vitro* by specific antigen. This reaction was not associated with circulating antibody and appeared to be an intimate property of the cells (2). Further studies indicated that only a few cells in a population need be sensitive for the migration of the whole population to be inhibited in the presence of antigen (3). In order to define the events occurring *in vitro*, it was necessary to obtain more precise information as to the nature of the sensitive cell. One approach to this problem was to study the effect of altering the surfaces of the sensitive cells.

In the present paper data will be presented to indicate that trypsinization of sensitive cells abolishes the inhibitory effect of specific antigen on their migration *in vitro*. This action of trypsin is prevented by the addition of soybean trypsin inhibitor. Evidence will also be presented to indicate that treatment of sensitive cells with trypsin may interfere temporarily with an immunologic capability of the cells.

Materials and Methods

Sensitization.—Delayed hypersensitivity was induced in Hartley strain guinea pigs by the injection of antigens incorporated in complete Freund's adjuvant (2).

Antigens.—Purified protein derivative (PPD), Merck, Sharpe and Dohme, West Point, Pennsylvania. Dinitrophenyl-bovine gamma globulin (DNP-BGG), kindly made available by Dr. Baruj Benacerraf.

Enzymes.—Trypsin[1] (1 and 3 times crystallized) was dissolved in Hanks'[2] balanced salt

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[1] Worthington Biochemical Corp., Freehold, New Jersey.

[2] Microbiological Associates, Bethesda.

solution in concentrations ranging from 10 μg/ml to 10 mg/ml. The enzyme in these concentrations is widely used in culture systems for the dissociation of cells from tissues and is not harmful to the cells (4, 5). The solutions were adjusted to pH 7.6 by the addition of isotonic $NaHCO_3$ and were Seitz filtered. 0.1 ml of packed cells were suspended in 2 ml of enzyme solution and incubated for 45 minutes at 37°C with frequent shaking. Following incubation, the cells were washed 3 times in balanced salt solution; the first wash contained soybean trypsin inhibitor[1] in excess of that necessary to combine with the trypsin available. In all experiments cells from the same pools were simultaneously incubated in balanced salt solution and served as controls.

Chymotrypsin[1] was prepared as above, in concentration of 1 mg/ml.

In experiments with ribonuclease[1] and desoxyribonuclease,[1] the enzymes were incorporated into the final tissue culture media and were not used in preincubation of the cells. The concentrations used were: RNAase, 50 and 1000 μg/ml; DNAase, 2 and 10 μg/ml.

Soybean Trypsin Inhibitor.—Trypsin was inactivated by the addition of soybean trypsin inhibitor as follows: 15 mg soy inhibitor to 5 mg/ml trypsin; 4000 μg soy inhibitor to 400 μg trypsin; 1000 μg soy inhibitor to 100 μg/ml trypsin. Cells were incubated in these mixtures as described above.

In Vitro Test for Delayed Hypersensitivity.—The methods used for placing the cells in culture have been described in detail elsewhere (2). In brief, the cells are washed, made up in tissue culture medium, centrifuged in capillary tubes, and the portion of the tubes containing the packed cells cut and placed in Mackaness type chambers. Chambers were filled with media containing specific antigen and sealed. Unless otherwise stated the concentrations of antigens were as follows: PPD, 15 μg/ml; DNP-BGG, 100 μg/ml. Control chambers contained the same media without antigen. The chambers were incubated for 24 hours at 37°C. During this period the cells migrated out of the tubes and onto the glass. The area of migration was projected, drawn, and measured with a planimeter. All data were calculated using the following formula:

$$\frac{\text{area of migration with antigen}}{\text{area of migration with no antigen}} \times 100 = \text{per cent migration with antigen}$$

Mixed Population Experiments.—The cells used to prepare mixed populations were harvested from animals exhibiting delayed hypersensitivity to DNP-BGG and from normal animals. A pool of sensitive cells and one of normal cells were prepared and each pool divided in half. One tube of sensitive and one of normal cells were placed in 1 per cent trypsin solution and incubated for 45 minutes at 37°C with frequent shaking. The remaining tubes of sensitive and normal cells were suspended in balanced salt solution and incubated in a similar manner. The suspensions were centrifuged and then washed 3 times in 100 volumes of balanced salt solution. The first wash contained 0.1 per cent soybean trypsin inhibitor, an amount in excess of that necessary to inactivate any trypsin remaining in the suspension. The mixed populations were made up to contain the following: population I, 50 per cent normal cells + 50 per cent sensitive cells; population II, 50 per cent trypsinized normal cells + 50 per cent sensitive cells; population III, 50 per cent normal cells + 50 per cent trypsinized sensitive cells.

The cell populations were suspended in tissue culture media, made up in capillary tubes, and assayed as described above.

Passive Transfer of Delayed Hypersensitivity.—A large pool of peritoneal cells from donors highly sensitive to tuberculin was collected and divided in half. One portion was incubated for 45 minutes at 37°C in trypsin solution 10 mg/ml (2 ml solution to each 0.1 ml packed cells). The other half was incubated in balanced salt solution and served as control. The cells

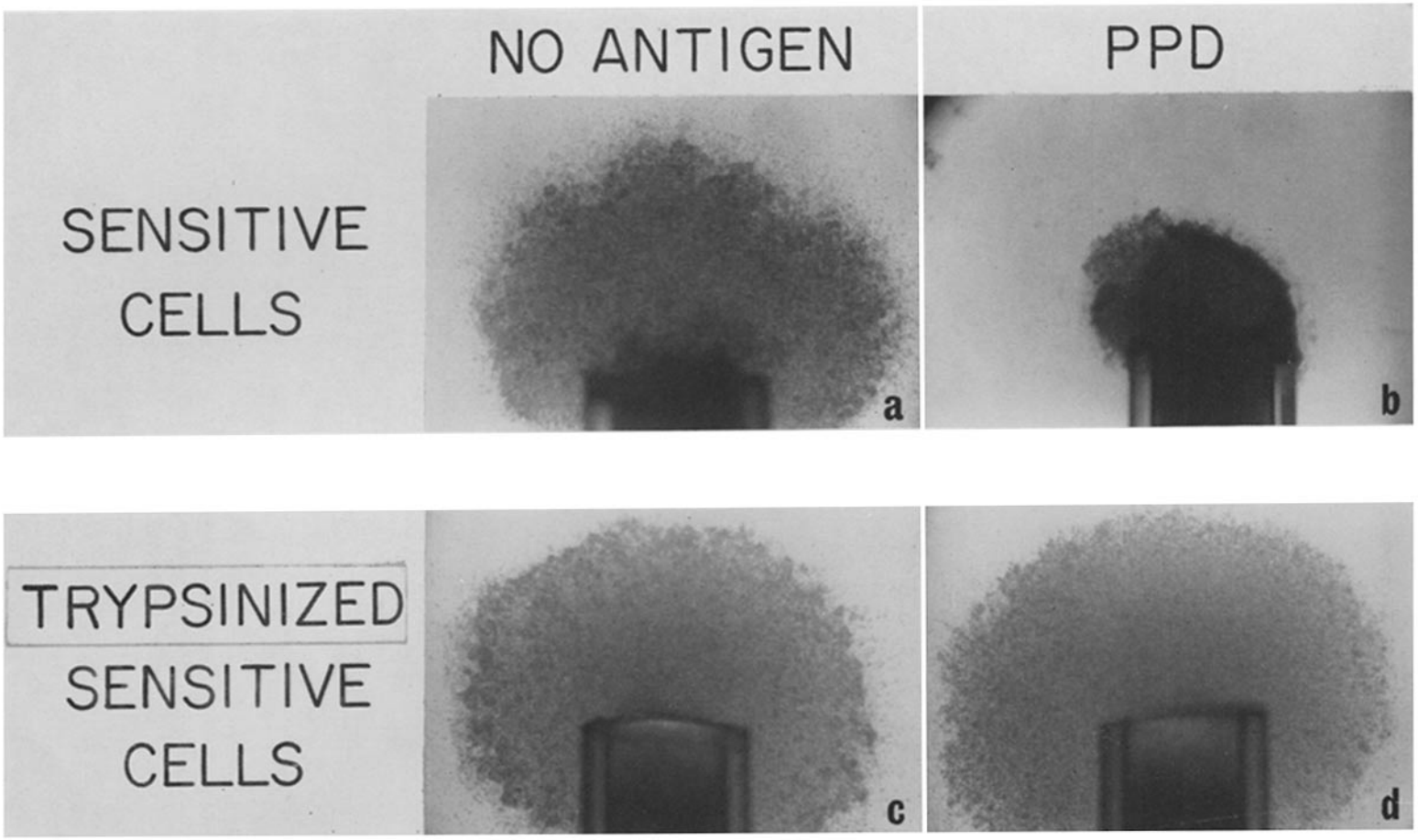

FIG. 1. The effect of trypsin on the migration of tuberculin-sensitive cells in the presence of antigen: (*a*) untreated sensitive cells migrating in normal media; (*b*) untreated sensitive cells inhibited by 15μg PPD/ml in the media; (*c*) trypsinized sensitive cells in normal media; (*d*) trypsinized sensitive cells in the presence of PPD.

TABLE I

The Effect of Trypsin on the Migration of Sensitive Cells in the Presence of Antigen

Migration with Antigen			
Cells from animals sensitive to DNP-BGG*		Cells from animals sensitive to tuberculin‡	
Control cells	Trypsinized§ cells	Control cells	Trypsinized§ cells
per cent	*per cent*	*per cent*	*per cent*
26	92	27.4	148
37‖	86.5	0	113¶
43‖	87	8.4	77
43.5	83.5	4.3	55**
47.5	103.5	33	111
45.5‖		44.2	96.5
46	112		
	103¶	15.9	96
17.2	85		
15.8	96.5		
40	84¶		
25.6	90.5		
34	98		
Average...35.1	93.4	19.0	99.5

* 100 μg DNP-BGG/ml in chambers.
‡ 15 μg PPD/ml in chambers.
§ 1 per cent trypsin (1 time, crystallized).
‖ 10 μg DNP/BGG/ml in chambers.
¶ 0.1 per cent trypsin (1 time, crystallized).
** 0.1 per cent trypsin (3 times, crystallized).

were washed 3 times, made up in balanced salt solution containing 5 units of heparin per ml and injected intravenously into normal guinea pigs. 1.0 ml of packed cells per animal were injected. The recipients were skin-tested with 20 μg PPD in 0.1 ml saline.

RESULTS AND DISCUSSION

Effect of Trypsinization on Normal and Sensitive Cells.—The *in vitro* migration of peritoneal cells from animals exhibiting delayed hypersensitivity was con-

sistently and markedly inhibited by specific antigen (2, 6). This inhibition by antigen was completely abolished by incubating the sensitive cells in trypsin prior to their being placed in culture (see Fig. 1). The trypsin was effective at concentrations of 1 or 10 mg/ml. At concentrations of 10 or 100 μg/ml, however, the abolition of inhibition was incomplete. The same results were obtained whether the cells were from animals with delayed hypersensitivity to tuberculin or to DNP-BGG. It is of interest that in three experiments 10 μg of DNP-BGG/ml was sufficient to inhibit the control cells from migrating while the trypsinized cells migrated well in 10 times that dosage (see Table I).

The addition of soybean trypsin inhibitor to the trypsin solution used for cell incubation nullified the effect of trypsin on the sensitive cells, indicating that trypsin is the effective agent rather than a contaminant of the enzyme preparagion (see Fig. 2). It is of interest that chymotrypsin produces the same effect as trypsin on the activity of the sensitive cells while RNAase and DNAase have no effect. These results suggest that trypsin digests a protein material from the surfaces of the sensitive cells which may temporarily desensitize them.

The effect of trypsin cannot be attributed to degradation of the protein antigens by trace amounts of trypsin remaining on the cells. Experiments showed that antigens incubated with normal trypsinized cells were later able to inhibit the migration of sensitive cells without loss of potency. It should also be noted that DNP has been used to mask the epsilon amino group of lysine, preventing the cleavage of a protein by trypsin at this point; therefore, it is unlikely that the active antigenic fragments of DNP-BGG would be destroyed by trypsin as used in our system. Finally, the experimental plan after the first two experiments provided that soybean trypsin inhibitor was present in the wash in tenfold excess to the amount of trypsin remaining in the cell suspension, a maneuver which guaranteed that no active trypsin remained on the cells when they were assayed in culture.

Mixed Population Experiments.—At this juncture it was apparent that the trypsin was acting on the cells rather than the antigen in the media. It was therefore important to determine whether trypsin was removing a material that was necessary to the immunologic capability of the sensitized cells, or, alternatively, was interfering in a non-specific manner with their ability to be inhibited by antigen. By mixing cells from sensitive and normal animals it has previously been shown that only a few cells in a population need be sensitive for the whole population to be inhibited by antigen (3). These findings suggested that antigen reacted specifically with the few sensitive cells, and this reaction then involved the surrounding non-sensitive cells so that the whole population failed to migrate. Whether the second step occurs because of the transfer of information from sensitive to normal cells or as a non-specific by-product of the antigen–sensitive cell interaction is unknown. The results were also consistent with the possibility that only a few cells in a population obtained from an animal exhibiting delayed hypersensitivity are specifically sensitive (3). Experiments

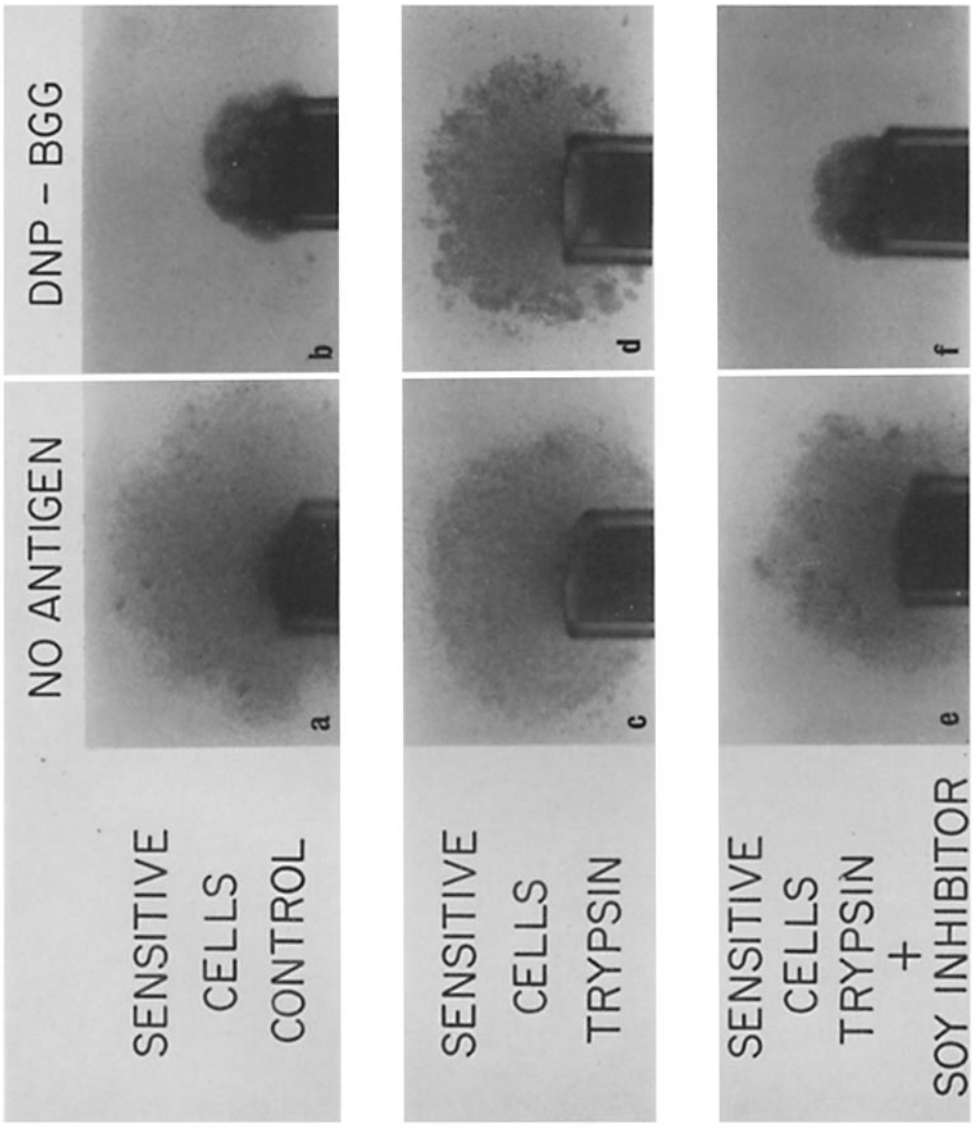

FIG. 2. The prevention of the action of trypsin on DNP-BGG-sensitive cells by soybean trypsin inhibitor: (*a*) untreated sensitive cells migrating in normal media; (*b*) untreated sensitive cells inhibited by 100 μg DNP-BGG/ml in the media; (*c*) sensitive cells pretreated with 0.5 per cent trypsin migrating in normal media; (*d*) sensitive cells pretreated with 0.5 per cent trypsin migrating well in media containing 100 μg DNP-BGG/ml; (*e*) sensitive cells pretreated with a mixture of 0.5 per cent trypsin + 1.5 per cent soybean trypsin inhibitor migrating in normal media; (*f*) sensitive cells pretreated with a mixture of 0.5 per cent trypsin + 1.5 per cent soybean trypsin inhibitor in media containing 100 μg DNP-BGG/ml.

were designed to determine whether trypsinization would interfere with the initial interaction between sensitive cells and antigen, or the resulting reaction involving normal cells. The results of the mixed population experiments are presented on Table II. Population I, which contained 50 per cent normal cells and 50 per cent sensitive cells was inhibited from migrating by antigen. Population II, which contained 50 per cent trypsinized normal cells and 50 per cent sensitive cells was also inhibited. Thus both populations in which the sensitive cells were untreated were inhibited from migrating by antigen. In contrast, the presence of specific antigen had no effect whatever on the migration of population III which contained trypsinized sensitive cells (see Fig. 3).

TABLE II

*The Effect of Trypsin in Mixed Population Experiments**

Experiment No.	Migration with antigen		
	Population I	Population II	Population III
	per cent	*per cent*	*per cent*
1	30	54.5	82.6
2	42.5	51.5	133
3	33.4	57	93
Average	35.6	54.3	102.8

* Population I, 50 per cent normal cells + 50 per cent sensitive cells; population II, 50 per cent trypsinized normal cells + 50 per cent sensitive cells; population III, 50 per cent normal cells + 50 per cent trypsinized sensitive cells.

The results of these experiments can be interpreted as follows: it is known that, in mixed populations, the sensitive cell-antigen interaction involves surrounding normal cells. If trypsin interferes with an immunologic capacity of the sensitive cells, the cells should be unable to initiate, by interacting with antigen, the chain of events that produces inhibition of migration. If, on the other hand, the specifically sensitive cells are *not* immunologically altered by trypsinization, they should be free to react with antigen and then involve the normal untrypsinized cells in the mixture, as they do in population I. Since there was no inhibition of migration when the sensitive cells were trypsinized, the conclusion that trypsinization of sensitive cells interferes with an immunologic function of those cells appears justified.

It should be noted that in the mixed population experiments, populations II and III contained an equal volume of trypsinized cells. Since population II was inhibited by antigen, the effect of trypsin cannot be attributed solely to a change in the cell's ability to adhere to glass. However, trypsinized cells occasionally migrated a little further in normal media than their untreated controls;

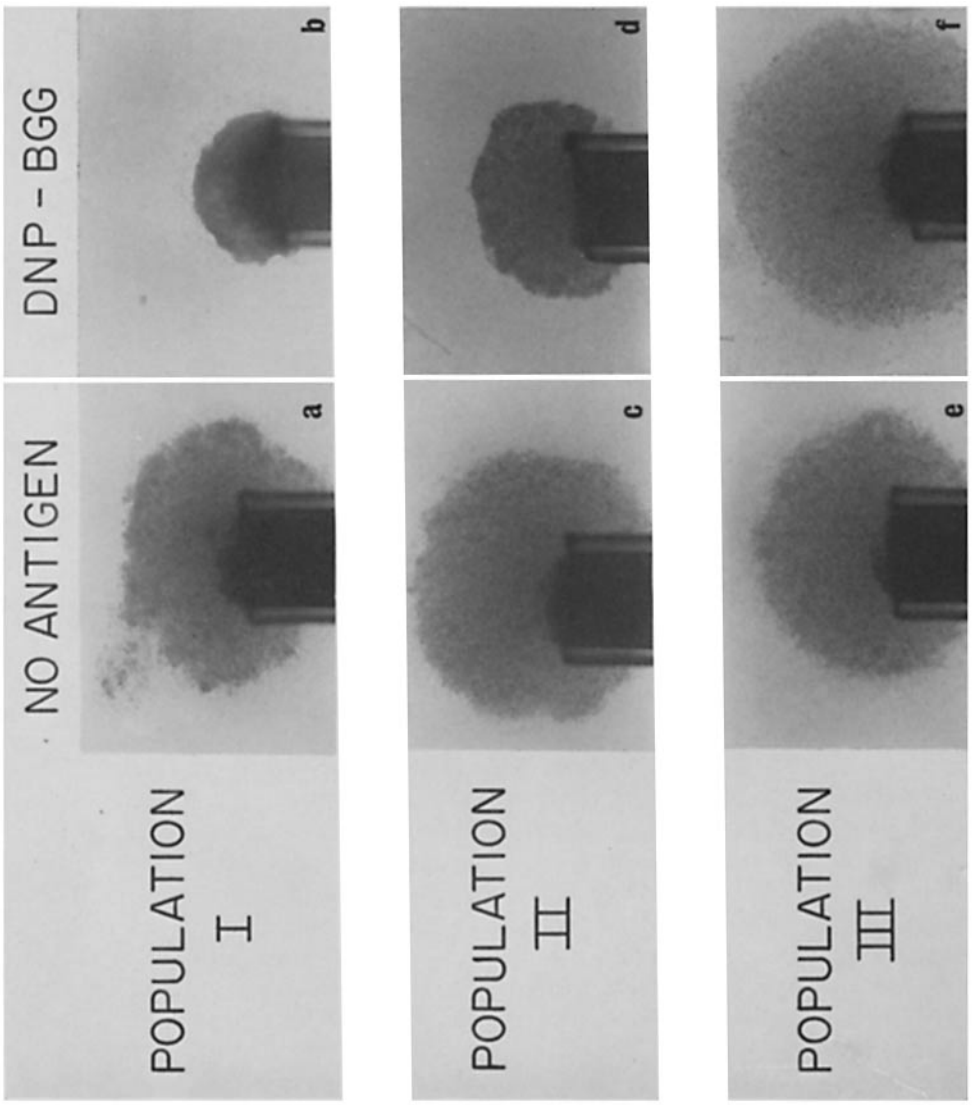

FIG. 3. *Experiments with mixtures of normal and sensitive cells.* Population I contained 50 per cent normal cells + 50 per cent sensitive cells: (*a*) migrating in normal media; (*b*) inhibited in the presence of 100 μg DNP-BGG/ml in the media. Population II contained 50 per cent normal trypsinized cells + 50 per cent sensitive cells: (*c*) migrating in normal media; (*d*) inhibited in media containing 100 μg DNP-BGG/ml. Population III contained 50 per cent normal cells + 50 per cent trypsinized sensitive cells: (*e*) migrating in normal media; (*f*) not inhibited in the presence of 100 μg DNP-BGG/ml in the media.

in the mixed populations containing trypsinized normal cells the populations were not inhibited to the same degree as the control groups. These observations suggest that there may also be a non-specific change in the surface properties of non-sensitive trypsinized cells which render them less amenable to the involvements that occur in the mixed population reactions.

Passive Transfer of Delayed Hypersensitivity with Trypsinized Cells.—In five experiments peritoneal cells from animals exquisitely sensitive to tuberculin were pooled and divided into two equal parts. The first part was trypsinized,

TABLE III

Passive Transfer of Delayed Hypersensitivity in Vivo with Trypsinized Peritoneal Cells

Experiment No.	Volume* of cells	Skin tests‡		
		Sensitive cells	Trypsinized sensitive cells	Control
1	0.6	5 x 5	7 x 7 2 x 2	0 2 x 2
2	0.8	15 x 20	18 x 18	0 0
3	1.1	22 x 16	8 x 5	0 2 x 2
4	1.1	22 x 13	16 x 12	0 4 x 4
5	0.96	Died	16 x 15 2 x 2	0 2 x 2

* Volume, milliliters of packed peritoneal exudate cells administered intravenously per animal.

‡ Animals skin-tested with 20 μg PPD at time of transfer. Erythema and induration measured in millimeters at 24 hours. Each skin test represents one animal.

the second incubated in balanced salt solution as control. These cells were injected into normal guinea pigs; recipients were skin-tested at the time of transfer. Trypsinized cells were consistently capable of passively transferring delayed hypersensitivity to tuberculin. Animals receiving the trypsinized sensitive cells gave positive skin tests although not always as large as those produced by control sensitive cells (see Table III).

This finding can be interpreted in at least two ways: (*a*) that trypsin alters a surface property of sensitive cells which is required for the *in vitro* reaction with antigen, but is not necessary for the cells to be capable of transferring delayed hypersensitivity, or (*b*) that the cells may resynthesize the material

removed by trypsin, allowing the transfer of sensitivity to proceed. The following results favor but do not prove the latter alternative.

In two experiments an aliquot of the trypsinized sensitive cells used to transfer were assayed *in vitro*, and, as expected, their migration was not inhibited by antigen. However, peritoneal cells harvested from the recipient animals 4 days after transfer were inhibited from migrating by antigen (see Table IV). It is currently thought that the passive transfer of delayed hypersensitivity in animals is associated with the presence in the recipient of sensitive cells from the

TABLE IV

In Vitro Assay of Trypsinized Sensitive Cells before and after Passive Transfer

Experiment No.	Type of cells used for transfer*	Volume of cells	Migration with antigen‡ of donor cells§		Skin test of recipients‖	Migration with antigen‡ of recipient cells¶
			Sensitive	Trypsinized sensitive		
		ml	*per cent*	*per cent*	*mm*	*per cent*
I	Sensitive	1.0	15.9	—	22 x 13	31
	Trypsinized sensitive	1.0	—	96	16 x 22	51.5
	No cells				0	118
	No cells		—	—	4 x 4	126
II	Sensitive	0.90	33	—	Died	—
	Trypsinized sensitive	0.96	—	111	16 x 15	42
	Trypsinized sensitive	0.96		111	2 x 2	129
	No cells		—	—	0	120
	No cells		—	—	2 x 2	96

* Guinea pigs weighed 426 ± 31 gm.

‡ 15 μg PPD/ml in chambers.

§ Aliquot of cells tested *in vitro* prior to transfer.

‖ Size of erythema and induration in millimeters 24 hours after 20 μg PPD intradermally.

¶ Peritoneal cells of recipients were tested 4 days after transfer.

donor, and sensitivity is lost when these cells have been rejected by a homograft reaction. The transfer is prolonged if carried out in an inbred strain (7). It is probable that the peritoneal cells obtained from the transferred recipient (which are inhibited by antigen *in vitro*) contain some of the trypsinized donor cells that prior to transfer could not be inhibited by antigen. These findings taken together suggest that the material removed from sensitive cells by trypsin is rapidly remade by the cells. Experiments are currently in progress attempting to study this recovery under *in vitro* conditions.

We have made numerous attempts to sensitize normal cells by incubating them in extracts of sensitive cells, but have been unable to do so. The possibility was considered that a proteolytic enzyme might be present in the cell ex-

tracts making it impossible to detect any transfer of information from sensitive extracts to normal cells. If such an enzyme were present in these extracts in significant amounts, it should be able to desensitize sensitive cells. It was found, however, that sensitive cells which had been incubated with extracts of normal cells were subsequently inhibited by antigen. Further, no trypsin or trypsin inhibitor was detected in the extracts; no other cathepsins were tested for.

GENERAL DISCUSSION

The data presented here suggest that trypsin removes a substance from the surface of sensitive cells which is closely associated with the immune capacity of those cells. It is likely that the cells are able to reproduce the material removed. Bloom, Hamilton, and Chase were able to prevent the transfer of delayed hypersensitivity by treating sensitive cells with mitomycin C or actinomycin D (8). It is possible that future studies will establish a relationship between the blocking of ribonucleic acid and protein synthesis in sensitive cells by metabolic inhibitors and the material removed from the surface of sensitive cells by trypsin. Although the nature of this postulated material is not known at present, there are several possibilities to be considered. The material removed by trypsin may provide a receptor for the attachment of antigen, or it may be a cell-bound immunoprotein. Although it has been shown that cytophilic antibody, as defined by Boyden and Sorkin (9), is not implicated in the *in vitro* system of delayed hypersensitivity (2), it is of interest that Sorkin (10) has shown that treatment of spleen cells with trypsin reduces their ability to absorb cytophilic antibody. It is also possible that trypsinization may remove a covering protein exposing other groups (such as mucopolysaccharides) thus changing the surface charge of the cell and altering its properties. From the data currently available, it is not possible to select which of these, or perhaps other, alternative mechanisms are operative.

SUMMARY

Peritoneal exudate cells from animals exhibiting delayed hypersensitivity are inhibited from migrating *in vitro* by specific antigen. This inhibition is completely abolished by pretreatment of the sensitive cells with trypsin. The action of trypsin is prevented by soybean trypsin inhibitor. The results of experiments with mixtures of normal and sensitive cells suggest that trypsin alters an immunologic capacity of the sensitive cells. Trypsinized sensitive cells are capable of passively transferring delayed hypersensitivity and peritoneal cells taken from recipient animals are inhibited from migrating *in vitro* by specific antigen. These results suggest that the cells rapidly resynthesize the material removed by trypsin. The possible nature of the material removed by trypsin is discussed.

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