THE CHEMOTACTIC EFFECT OF MIXTURES OF ANTIBODY AND ANTIGEN ON POLYMORPHONUCLEAR LEUCOCYTES

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Although it has been known for many years that some motile plant and animal cells will move towards or away from foci from which certain chemical substances are diffusing, we are very ignorant of the mechanisms involved in these chemotactic responses. This applies particularly to chemotaxis among metazoan cells. Even in the case of mammalian leucocytes, which have long been known to migrate actively towards clumps of bacteria, starch grains, etc., (see references 1, 2 and 3), information is lacking on the chemical nature of the substances to which the cells are responding.

Our ignorance in this field is largely attributable to the lack of suitable techniques for testing soluble substances for chemotactic activity. While it is a relatively simple matter to show *in vitro* that leucocytes migrate towards a clump of bacilli, it is very hard to show whether a given soluble bacterial product is chemotactic. Attempts have been made to estimate the chemotactic effect of soluble substances by absorbing them onto various sorts of particles which are then tested for a capacity to attract leucocytes. This technique is very imprecise and it is impossible to use it for making quantitative comparisons between different substances for chemotactic activity.

In connection with some studies in our laboratory on the mechanisms by which leucocytes discriminate between indigenous and foreign matter, we have developed a method which seems to provide a more satisfactory means of estimating the relative chemotactic effect of soluble substances. The technique, which is described in detail below, involves the use of a perspex chamber which is separated into two compartments by a filter membrane of such pore size that leucocytes cannot pass through it except by active migration. The cells are allowed to settle on one side of the membrane, while a solution of the substance to be tested for chemotactic activity is placed on the other. After incubation for a certain time, the filter membrane is fixed, stained, and cleared, and the number of cells which have migrated through to the far side is counted microscopically.

Substances tested for chemotactic activity in this technique have so far been limited mainly to preparations of proteins and of non-dialyzable macromole-

cules obtained from the body fluids of various species of animals and from plant and bacterial sources. The cell mostly studied has been the rabbit polymorphonuclear leucocyte. In general it has been found that the further removed, in the phylogenetic sense, the biological source of the test material is, the more active is it likely to be as a chemotactic agent for the rabbit cells. For example, in a medium containing normal rabbit serum, such substances as human serum albumin (H.S.A.) and bovine serum albumin show only a weak chemotactic activity or none at all, whereas plant and bacterial protein preparations are usually strongly chemotactic.

Early in these investigations it was noted that substances like H.S.A., which normally do not attract leucocytes, elicit a strong chemotactic response in the presence of the corresponding specific antiserum. This observation led to some experiments on the chemotactic activity of antibody-antigen complexes. Some of these experiments are reported in the present paper which also contains a full description of the experimental procedure for assessing chemotaxis.

Materials and Methods

Rabbits used for the preparation of antisera and as donors of normal serum and of cells were of mixed stock and both sexes. They weighed between 2000 and 3000 gm.

Rabbit Anti-H.S.A. Serum.—Two rabbits received 10 weekly intravenous injections of 2 mg human serum albumin (Nutritional Biochemicals Corporation, Cleveland) absorbed onto aluminum hydroxide. After a rest period of 5 weeks they were reinjected as previously. 7 days later blood was taken from the ear vein. The two sera were pooled. The pool contained 0.12 mg antibody nitrogen per ml (precipitable with H.S.A.).

Rabbit Anti-Ovalbumin Serum.—One rabbit received 10 weekly intravenous injections of 10 mg ovalbumin in 1 ml saline. After a rest period of 5 weeks the rabbit was reinjected with an identical dose of ovalbumin intravenously. Blood was taken from the ear vein 7 days later. A good precipitating serum was obtained. No estimation of antibody nitrogen was made.

Polymorphonuclear leucocytes were obtained from the peritoneal cavity of rabbits by the technique described by Hirsch and Church (4). 150 mg oyster glycogen (British Drug Houses Ltd., Poole, England) was injected intraperitoneally and the exudate was collected 3½ hours later. The exudates contained over 98 per cent polymorphonuclear leucocytes.

The cells were centrifuged lightly and resuspended in medium (see below) to a concentration of 1.5×10^6/ml.

Rabbits were not injected with glycogen more frequently than once every 3 weeks.

Medium.—The basic medium used in these experiments was 20 per cent normal rabbit serum in Hanks' solution (as given by Weller and Enders) (5) which contained 50 units of penicillin and 50 units of streptomycin per ml. The normal rabbit serum was a pool of sera from eight normal rabbits. It was kept stored at −50° in 5 to 10 ml quantities.

In some experiments special sera (*e.g.* anti-H.S.A. serum) were included in the medium, in which case proportionately less normal rabbit serum was added. Full details of the different mixtures are given with the descriptions of the individual experiments. "Hanks' soln.", in these descriptions, refers to Hanks' solution containing penicillin and streptomycin as described above. "N.R.S." means normal rabbit serum.

Technique for Estimating Chemotaxis.—The apparatus is a perspex chamber (Fig. 1) which consists essentially of two separate compartments (*A* and *B*) separated during the course

of an experiment by a Millipore (Millipore Filter Corporation, Bedford, Massachusetts) filter membrane (13 mm diameter) (*D*) held in position by a double ring (*C* and *E*) of perspex.

When the apparatus is to be assembled, the lower surface of ring *E* is lightly smeared with sterile petroleum jelly and is then placed in the socket between the two compartments *A* and *B*. The filter membrane is put in the cup-like ring *E*, and the ring *C* is pushed down on top of the membrane to hold it firmly in position.

When both the cell suspension and the test solution are ready, 1.5 ml of test solution (or normal medium in the case of the controls) is run into compartment *A*, the apparatus being tipped slightly to avoid bubbles becoming trapped beneath the filter membrane. 3.6 ml of cell suspension is then pipetted into compartment *B* at the same time as the rest (1.5 ml) of the test solution is run into compartment *A*. The apparatus is placed at 37°C in a moist atmosphere containing 5 per cent CO_2 in air for 3 hours.

After this period of incubation, the double ring (*C* and *E*) is taken out of the apparatus. (The groove on the outside of ring *E* is to facilitate removal with specially hooked forceps.) Rings *C* and *E* are then separated and the filter membrane is picked out with a pair of fine forceps. It is then rinsed very gently in Hanks' solution and then treated as follows:—

Methanol, 5 seconds. Rinse in distilled water. Erhlich's hematoxylin, 5 minutes. Rinse in distilled water. 1 per cent acid alcohol, 1 minute. Rinse in distilled water. Blueing agent (2 gm sodium bicarbonate, 20 gm magnesium sulphate in 1 litre distilled water), 2 minutes. Rinse in distilled water. 70 per cent ethanol, 2 minutes. 95 per cent ethanol, 2 minutes. Absolute ethanol, 3 minutes. Absolute ethanol, 3 minutes. Clear in xylene. It is important not to leave the membrane in methanol or ethanol too long or damage will occur to the filter.

Assessment of Results.—The millipore membranes are examined microscopically. The membrane, cleared with xylene, is transparent and the cells can be visualized at all levels; however, in the experiments which follow only cells reaching the far side of the membrane have been scored. For counting the cells the × 40 objective and × 10 ocular were used. The number of cells was counted in each of 10 randomly selected fields. The results in the tables represent the average count per field.

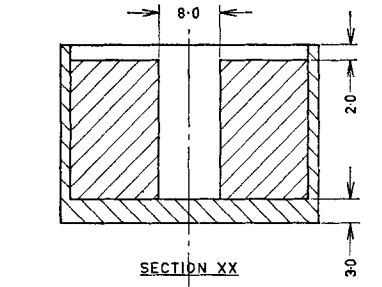
EXPERIMENTAL

Migration through Membranes towards Products of Myocobacterium tuberculosis.—The following experiment illustrates the principle of the method used for estimating the chemotactic activity of soluble substances.

Nine chambers were used. In three chambers normal medium was placed in both compartments (set *a*). Another three chambers contained normal medium in the cell compartment, and medium containing tuberculin in the test compartment (set *b*). In a final set medium containing tuberculin was placed in both compartments (set *c*).

The results, given in Table I, show that when tuberculin was present in the test compartment only (set *b*), the number of cells found on the far side of the membrane was substantially higher than in the control (set *a*) in which both compartments contained normal medium.

In set *c*, in which tuberculin was present in both compartments, migration was similar to that in set *a*. This indicates that in set *b* the cells are, in fact, responding by active migration towards the medium containing the higher concentration of tuberculin, and that the effect of the tuberculin is not merely due to an increase in the rate of random movement of the cells in the membrane.



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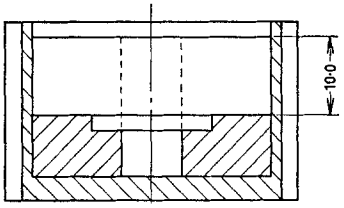

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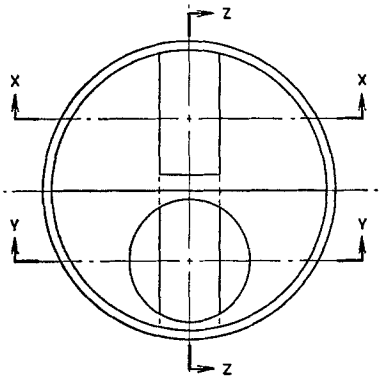

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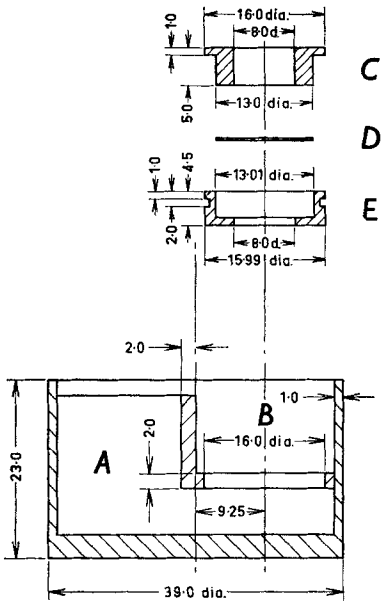

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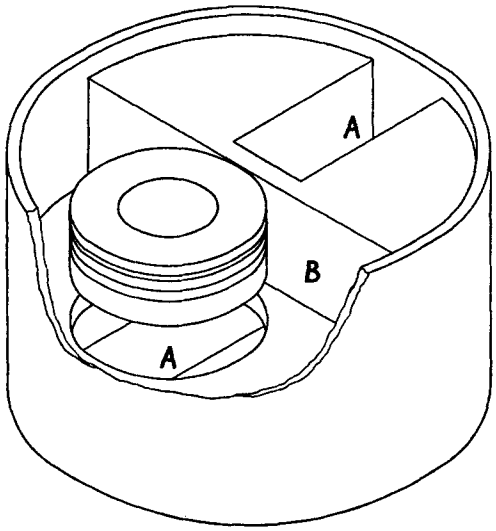

The Chemotactic Effect of Mixtures of Antibody and Antigen.—While bacterial and plant products and macromolecular preparations from the body fluids of animals far removed from the rabbit phylogenetically tend to have marked chemotactic effect on rabbit granulocytes, not all "foreign" proteins produce this response. In fact various mammalian serum albumin preparations and also ovalbumin have given negative results when tested in experiments identical in principle with that described above.

TABLE I

Chemotactic Activity of Crude Tuberculoprotein

Test solution in compartment B	Medium in compartment A	Migration
		cells/field
Set *a*		
(1) Normal medium	Normal medium	2
(2) " "	" "	1
(3) " "	" "	1
Set *b*		
(4) Normal medium	Tuberculoprotein 0.03 mg/ml	47
(5) " "	" " "	57
(6) " "	" " "	45
Set *c*		
(7) Tuberculoprotein 0.03 mg/ml	Tuberculoprotein 0.03 mg/ml	1
(8) " " "	" " "	3
(9) " " "	" " "	1

In the following experiment the effect is tested of adding specific antiserum to two such chemotactically inactive preparations, human serum albumin and ovalbumin.

Nine chambers were set up. In each case the cell compartment contained leucocytes in normal medium. The mixtures in the test compartments were as follows:—

(1) 0.6 ml N.R.S. + 2.4 ml Hanks' soln.
(2) 0.6 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.
(3) 0.6 ml N.R.S. + 0.2 ml ovalbumin 0.01 mg/ml + 2.2 ml Hanks' soln.
(4) 0.2 ml anti-H.S.A. + 0.4 ml N.R.S. + 2.4 ml Hanks' soln.
(5) 0.2 ml anti-H.S.A. + 0.4 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.
(6) 0.2 ml anti-H.S.A. + 0.4 ml N.R.S. + 0.2 ml ovalbumin 0.01 mg/ml + 2.2 ml Hanks' soln.
(7) 0.2 ml anti-ovalbumin + 0.4 ml N.R.S. + 2.4 ml Hanks' soln.
(8) 0.2 ml anti-ovalbumin + 0.4 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.
(9) 0.2 ml anti-ovalbumin + 0.4 ml N.R.S. + 0.2 ml ovalbumin 0.01 mg/ml + 2.2 ml Hanks' soln.

The results of this experiment are given in Table II.

The results in Table II show that of all the nine mixtures, only two promoted a marked migration of leucocytes through the membrane. The two chemotactic mixtures were ovalbumin and H.S.A. mixed with the homologous antisera. Both antigens gave negative results when tested in normal serum or when mixed with the heterologous antiserum.

TABLE II

Chemotactic Activity of H.S.A., of Ovalbumin and of Mixtures of These Antigens with the Corresponding Antisera

Serum	Antigen added*		
	No antigen	H.S.A.	Ovalbumin
N.R.S.	(1) 2	(2) 1	(3) 2
Anti-H.S.A.	(4) 3	(5) 97	(6) 2
Anti-ovalbumin	(7) 1	(8) 2	(9) 47

* The figures not in parentheses refer to the average number of cells per field. The figures in parentheses indicate the number of the chamber as follows:—

(1) 0.6 ml N.R.S. + 2.4 ml Hanks' soln.

(2) 0.6 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.

(3) 0.6 ml N.R.S. + 0.2 ml ovalbumin 0.01 mg/ml + 2.2 ml Hanks' soln.

(4) 0.2 ml anti-H.S.A. + 0.4 ml N.R.S. + 2.4 ml Hanks' soln.

(5) 0.2 ml anti-H.S.A. + 0.4 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.

(6) 0.2 ml anti-H.S.A. + 0.4 ml N.R.S. + 0.2 ml ovalbumin 0.01 mg/ml + 2.2 ml Hanks' soln.

(7) 0.2 ml anti-ovalbumin + 0.4 ml N.R.S. + 2.4 ml Hanks' soln.

(8) 0.2 ml anti-ovalbumin + 0.4 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.

(9) 0.2 ml anti-ovalbumin + 0.4 ml N.R.S. + 0.2 ml ovalbumin 0.01 mg/ml + Hanks' soln.

Effect of Varying Antigen Concentrations in the Presence of a Fixed Amount of Antibody.—In this experiment mixtures of different concentrations of H.S.A. and a fixed amount of anti-H.S.A. were tested for chemotactic activity.

Ten chambers were set up. In each case the cell compartment contained leucocytes in normal medium. The mixtures in the test compartments were as follows:—

(1) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 1.6 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.

(2) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.4 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.

(3) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.1 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.

(4) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.025 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.

(5) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.006 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.

(6) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.0015 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(7) 0.6 ml N.R.S. + 0.2 ml H.S.A. 1.6 mg/ml + 2.2 ml Hanks' soln.
(8) 0.6 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.
(9) 0.6 ml N.R.S. + 0.2 ml H.S.A. 0.006 mg/ml + 2.2 ml Hanks' soln.
(10) 3.0 ml normal medium.

TABLE III

Effect of Antigen Concentration on the Chemotactic Activity of Antibody-Antigen Mixtures

Concentration of H.S.A.* added	Migration	
	With antiserum	Without antiserum
mg/ml	*cells/field*	*cells/field*
1.6	(1)‡ 25	(7) 11
0.4	(2) 230	
0.1	(3) 450	(8) 4
0.025	(4) 355	
0.006	(5) 296	
0.0015	(6) 45	(9) 4
No H.S.A.		(10) 3

* The final concentration of antigen in the test medium is $\frac{1}{15}$ of the concentration of antigen added.

‡ The figures in parentheses refer to the numbers of the chambers with mixtures as follows:—
(1) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 1.6 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(2) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.4 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(3) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.1 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(4) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.025 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(5) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.006 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(6) 0.2 ml anti-H.S.A. serum + 0.2 ml H.S.A. 0.0015 mg/ml + 0.4 ml N.R.S. + 2.2 ml Hanks' soln.
(7) 0.6 ml N.R.S. + 0.2 ml H.S.A. 1.6 mg/ml + 2.2 ml Hanks' soln.
(8) 0.6 ml N.R.S. + 0.2 ml H.S.A. 0.1 mg/ml + 2.2 ml Hanks' soln.
(9) 0.6 ml N.R.S. + 0.2 ml H.S.A. 0.006 mg/ml + 2.2 ml Hanks' soln.
(10) 3.0 ml normal medium.

The results of this experiment (Table III) show that, for a given concentration of antibody, there is an optimum concentration of antigen for producing maximum chemotactic effect. The mixture which caused most migration was that in which the most precipitate was formed. Less migration occurred in the case of the mixtures in the region of antigen excess.

The possibility was considered that the relatively slight migration in the region of antigen excess might be due to the presence in this region of an excessively high concentration of chemotactically active molecules, so that all receptors on the cells were quickly satisfied and the cells were thus unable to sense the concentration gradient. That this was not the explanation was shown by the finding that dilutions (¼ and 1/16) in normal medium of the mixture in antigen excess (No. 1 in the above experiment) were possessed of even less chemotactic activity than the undiluted mixture.

The Chemotactic Activity of Suspernatants after Centrifugation of Antibody-Antigen Mixtures.—Although it seemed unlikely that the chemotactic effect was due to very small aggregates of specific precipitate passing through the filter membrane (or forming within the membrane) and acting directly on the cells, the fact that maximal migration occurred with mixtures made in the region of equivalence was in keeping with this interpretation. The following experiment was designed to test the chemotactic effect of the antigen-antiserum mixture after removal of the specific precipitate.

An antibody-antigen precipitate was prepared by mixing 0.3 ml of anti-H.S.A. serum, 0.3 ml H.S.A. at 0.1 mg/ml, 0.6 ml N.R.S. and 3.3 ml Hanks' solution. The mixture was incubated for 1 hour at 37°C and then centrifuged at 2,000 *g* for 15 minutes. The supernatant fluid (S1) was stored at −50°C overnight. The precipitate was resuspended in 4.5 ml of cold medium and kept at 0°–4°C overnight. The following morning the mixture was centrifuged at 2,000 *g* for 10 minutes. The supernatant (S2) was kept and the precipitate was resuspended in 4.5 ml medium. The mixture was again incubated at 37°C for 1 hour and then centrifuged at 2,000 *g* for 15 minutes. The supernatant fluid (S3) was also kept and the deposit was resuspended in 4.5 ml medium. 3 ml of each of the supernatants and of the resuspended final precipitate were tested for chemotactic activity in the usual way. Controls included normal medium which had been stored overnight at −50°C (No. 5), normal medium stored at 0°–4°C overnight (No. 6) and normal medium stored at −50°C overnight and incubated the following morning at 37°C for 1 hour.

The results of this experiment are given in Table IV. The supernatant (1) obtained by centrifugation of the antigen-antibody precipitate after incubation for 1 hour possesses considerable chemotactic activity. The next supernatant (2), which was taken off after 17 hours in the cold, produced negligible migration of the cells. However, the supernatant (3) which followed reincubation at 37°C of the precipitate in fresh medium on the 2nd day was again active. The precipitate itself, when tested in fresh medium, was also active and caused the migration of over twice as many cells as did the two active supernatants. (It must be borne in mind when comparing these figures that the precipitate was in contact with each of the supernatants for only 1 hour, and in contact with the medium in which it was itself tested for a period of 3 hours).

These results suggest that at least a part of the chemotactic activity of the antibody-antigen mixtures is due to substances in solution, distinct from the specific aggregation of antibody and antigen. This experiment does not answer

the question whether the chemotactic substances are soluble antibody-antigen complexes (perhaps formed during incubation by dissociation of some of the specific precipitate) or whether the interaction of antibody and antigen results in the liberation of a soluble chemotactic substance.

It should be noted that, although in this particular experiment the mere incubation at 37°C of medium (containing, of course, normal rabbit serum) did not confer upon it any chemotactic activity, in other experiments medium which has been incubated at 37° for a time has sometimes been found to stimulate some migration of leucocytes, but only to levels of about two times that of the controls with unincubated normal medium.

The Effects of Inactivation of Antibody-Antigen Mixtures at 56°C before and after Incubation at 37°C.—Some experiments were performed to test whether

TABLE IV

The Chemotactic Activity of Supernatants after Centrifugation of Antibody-Antigen Mixtures

	Migration
	cells/field
(1) Supernatant 1. (after 1 hr. at 37°C 1st day)	107
(2) Supernatant 2. (after overnight at 0°–4°C)	19
(3) Supernatant 3. (after 1 hr. at 37°C. 2nd day)	136
(4) Resuspended precipitate	346
(5) Control. Normal medium	4
(6) Control. Normal medium (stored at 0°–4°C overnight)	6
(7) Control. Normal medium (incubated 1 hr. 37°C)	6

the chemotactic effect of antibody-antigen mixtures could be demonstrated when all the serum in the medium (including antiserum) had been previously inactivated by treatment at 56°C for 30 minutes. The results showed that when only inactivated serum was used in the medium the "random" migration of the granulocytes in the controls was at least as great as, and often slightly greater, than that which occurred when the medium contained fresh serum. Although the cells were thus obviously capable of migration in medium containing only inactivated serum, the inclusion of antiserum (inactivated) and antigen in the test compartment did not result in any greater migration than in the controls. Nevertheless, washed specific precipitates made by mixing antigen with inactivated antiserum were strongly chemotactic when added to fresh serum.

Thus, although the antigen and the specific antibody were stable at 56°C, they exhibited no chemotactic activity when tested in medium which had been treated at that temperature. The natural conclusion from these results is that a non-specific heat-labile factor (or factors) must also be involved in the production of the chemotactic response.

It was postulated at this stage that the antibody-antigen complex might activate a heat-labile component of serum (perhaps complement) and that enzymatic activity ensues which results in the production of chemotactically active substances which act directly on the cells. These chemotactic products might be either heat-labile or heat-stable, and the following experiment was designed to test the latter possibility.

In this experiment antibody and antigen were allowed to interact in fresh serum at 37°C for 1 hour. After this period of incubation the mixtures were inactivated at 56°C for 30 minutes.

TABLE V

Effect of Treatment at 56°C before and after Incubation of Antibody-Antigen Mixtures at 37°C

	Migration
	cells/field
(1) Mixture A* (antibody and antigen inactivated after incubation at 37°C)	268
(2) Mixture B (N.R.S. and antigen inactivated after incubation at 37°C)	7
(3) Mixture C (antibody and antigen inactivated before incubation at 37°C)	14
(4) Mixture D (N.R.S. and antigen inactivated before incubation at 37°C)	5
(5) Normal medium	8

* The four mixtures were made as follows:—

Mixture A. 0.5 ml anti-H.S.A. (inactivated) + 0.5 ml H.S.A. 0.1 mg/ml + 1.0 ml N.R.S. (inactivated) + 5.5 ml Hanks' soln.

Mixture B. 1.5 ml N.R.S. (inactivated) + 0.5 ml H.S.A. 0.1 mg/ml + 5.5 ml Hanks' soln.

Mixture C. 0.5 ml anti-H.S.A. inactivated) + 0.5 ml H.S.A. 0.1 mg/ml + 1.0 ml N.R.S. (fresh) + 5.5 ml Hanks' soln.

Mixture D. 1.5 ml N.R.S. (fresh) + 0.5 ml H.S.A. 0.1 mg/ml + 5.5 ml Hanks' soln.

Controls included similar preparations containing antigen but no antiserum, and mixtures of antigen and antiserum in which all serum components of the mixture were inactivated prior to incubation at 37°C.

The following four mixtures were made:—

A. 0.5 ml anti-H.S.A. (inactivated) + 0.5 ml H.S.A. 0.1 mg/ml + 1.0 ml N.R.S. (inactivated) + 5.5 ml Hanks' soln.

B. 1.5 ml N.R.S. (inactivated) + 0.5 ml H.S.A. 0.1 mg/ml + 5.5 ml Hanks' soln.

C. 0.5 ml anti-H.S.A. (inactivated) + 0.5 ml H.S.A. 0.1 mg/ml + 1.0 ml N.R.S. (fresh) + 5.5 ml Hanks' soln.

D. 1.5 ml N.R.S. (fresh) + 0.5 ml H.S.A. 0.1 mg/ml + 5.5 ml Hanks' soln.

All mixtures were incubated at 37°C for 1 hour. C and D were then inactivated by treating at 56°C for 30 minutes. 3 ml of each mixture was then tested for chemotactic activity by the usual procedure.

The medium in this experiment consisted of 20 per cent *inactivated* (56°C 30 minutes) normal rabbit serum instead of fresh serum. The results of this experiment are given in Table V.

The results show that whereas the antibody-antigen mixture which was inactivated *before* the period of incubation had a negligible chemotactic effect, the mixture which was inactivated *after* the incubation period was strongly chemotactic.

These results are consistent with the interpretation that the antibody-antigen complex activates a heat-labile substance, perhaps an enzyme, which causes the production of a heat-stable chemotactic agent which acts directly on the cells.

DISCUSSION

Many types of cell in the metazoan body are probably capable of such characteristics as adhesiveness, phagocytosis, ameboid movement and directional migration. The differences in the cells, from this behavioral standpoint, lie less in what they are capable of doing than in what particular stimuli induce them to do it. However, in very few instances indeed is information available on the precise nature of the factors which provoke the different sorts of behaviour in the various cell types.

Even in the case of mammalian leucocytes, which are relatively easy to handle *in vitro* and which have received a great deal of experimental attention, we are very ignorant of the factors which control the characteristic activities of these cells. By what mechanism, for instance, does the polymorphonuclear leucocyte discern differences between healthy indigenous cells of the host on the one hand and clumps of bacteria on the other, so that it actively migrates towards, and ultimately engulfs the latter, while ignoring the former? The same question can be asked in respect of the ameboid cells of all metazoa.

Although good evidence is available that the leucocytes respond by active migration towards chemical substances diffusing from clumps of bacteria, starch grains, etc., (see references 1, 2 and 3), information on the actual nature of the chemotactic stimuli is almost completely lacking. The general opinion, based on very little evidence, seems to be that the cells react to general classes of chemical substances, such as amino acids, or polysaccharides which are thought to be given off by the attractive particles. The possibility is worth considering, however, that the chemotactic response is a more selective process than this, and that the phagocytes, like the cells responsible for antibody production, respond to the presence in their environment of macromolecules which differ in structure from those normally present in the host.

One of the main difficulties in studying chemotaxis experimentally has been the lack of adequate techniques. None of the procedures reported in the literature provide a satisfactory means of estimating quantitatively the chemotactic effects of different substances in solution. The method described in this paper was designed to overcome the need for such a technique.

The experiments reported above were carried out following the observation

that H.S.A., which had little or no chemotactic activity when tested in medium containing normal serum, caused marked leucocytic migration in the presence of rabbit antibodies to H.S.A. At first it was assumed that the antibody-antigen complexes were exerting a direct influence on the leucocytes. However, the observation that the maximum effect occurred in mixtures of antibody and antigen in regions of equivalence, where maximum precipitation occurred, was rather hard to explain on this basis, since the amount of complex making contact with the cells in such mixtures would be less than in regions of antigen excess.

It was then found that if antibody-antigen mixtures in the equivalence zone were incubated at 37°C and then centrifuged, the resultant supernatants were strongly chemotactic. This observation suggested that the agent acting on the leucocyte might not be the antibody-antigen complex itself, but rather some by-product of the interaction of antibody and antigen. Some further experiments were set up with this possibility in mind, and the following conclusions can be drawn from the results. Firstly, it is apparent that the leucocytes are capable of responding to a chemotactic stimulus by active directional migration in the presence of inactivated as well as fresh serum. Secondly, the interaction of antibody and antigen results in the production of a heat-stable chemotactic substance (or substances). Thirdly, the chemotactic substance is not produced when antibody and antigen are allowed to interact in inactivated serum.

The simplest interpretation of the facts is that antibody and antigen combine to form a complex which interacts with a heat-labile substance (probably an enzyme, perhaps a component of complement), and that as a consequence of this interaction a heat-stable substance is produced which has a direct chemotactic influence on the leucocytes. This interpretation may be represented as follows:—

$$\underset{\text{(stable at 56°C)}}{\text{Ab} + \text{Ag}} \rightarrow \text{[Ab. Ag] complex}$$

$$\underset{\text{(labile at 56°C)}}{\text{inactive enzyme}} \xrightarrow{\text{[Ab. Ag]}} \text{active enzyme}$$

$$\text{precursor of chemotactic substance} \xrightarrow{\text{active enzyme}} \underset{\text{(stable at 56°C)}}{\text{chemotactic substance}}$$

Needless to say, the situation may well turn out to be much more complex than this. Nevertheless this scheme can be used as a basis for further work, the first steps in which will be an attempt to characterise and identify the chemotactic substance.

The effect of homologous antibody on the chemotactic effect of antigens such as H.S.A. and ovalbumin, which in normal serum usually have no demonstrable activity, raises the question whether the chemotactic effect of preparations

which are chemotactic in normal serum such as tuberculoproteins, insect blood proteins etc. (6) may not also be dependent on serum factors. Investigation of this question is in progress, and the findings to date are compatible with this interpretation. The fact that bacterial products are generally more chemotactic in normal serum than, for example, is human serum albumin, may merely reflect the existence in the bacterial preparations of a much greater number of macromolecular configurations different from those of the host's own components. There is thus more interaction of "natural antibodies" with the bacterial products than with purer products from more closely related species.

It is reasonable to suppose that antibody-antigen complexes can exert a chemotactic effect *in vivo* as well as *in vitro*. This has obvious implications in relation to specific acquired resistance to natural microbial infections; it may also offer an explanation for the massive polymorphonuclear infiltration which follows intradermal injection of antigen in the Arthus reaction.

It can be hoped that procedures similar to that described in this paper for the estimation of chemotactic influences on granulocytes may also prove useful for study of other cell types, both of metazoal and protozoal origin. We have noted, for instance, that lymphocytes migrate actively through Millipore filters *in vitro*, an observation which has previously been made *in vivo* (Shelton and Rice, 7). It would be very worthwhile to try to identify the chemotactic factors which influence these cells, perhaps taking as a basis for research Pulvertaft's (8) demonstration of the extraordinary interest which lymphocytes show in cells which are undergoing mitosis.

This work was carried out with the technical assistance of Mrs. Elizabeth Orosz.

SUMMARY

An *in vitro* technique is described for assessing the chemotactic activity of soluble substances on motile cells.

Antibody-antigen mixtures when incubated (37°C) in medium containing fresh (*i.e.* non-inactivated) normal rabbit serum exert a strong chemotactic effect on rabbit polymorphonuclear leucocytes.

Results are described which indicate that, when antibody-antigen complexes are incubated (37°C) in fresh serum, a heat-stable (56°C) substance (or substances) is produced which acts directly as a chemotactic stimulus on the polymorphs. This heat-stable chemotactic substance is not produced when antibody-antigen complexes are incubated in serum which has been heated at 56°C for 30 minutes.

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