Immunology, 1965, **9**, 441.

Studies on Chemotaxis

II. THE SIGNIFICANCE OF NORMAL SERA FOR CHEMOTAXIS INDUCED BY VARIOUS AGENTS

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(*Received* 8*th February* 1965)

Summary. Fresh normal sera of rabbits, guinea-pigs, pigs, calves, sheep and men when incubated with complexes of human serum albumin and rabbit antibody induce chemotaxis of rabbit granulocytes *in vitro*. No chemotaxis was observed when such sera were heated prior to the addition of antigen–antibody complexes. Incubation of fresh normal rabbit serum with heat-aggregated γ-globulin, PPD, glycogen, *Proteus* endotoxin or heat-killed *Staphylococcus albus* results in formation of chemotactic factor(s). No chemotactic activity is found if these agents are incubated in inactivated normal rabbit serum or in serum-free medium. In contrast, the chemotactic activity of Witte's peptone does not depend on the presence of serum.

INTRODUCTION

Convincing evidence has been presented by Boyden (1962) that the interaction of antigen–antibody complexes with fresh rabbit serum containing heat-labile components leads to the formation of heat-stable chemotactic mediator(s). We have recently further shown that the chemotactic effect of heat-aggregated human γ-globulin similarly depends on the presence of heat-labile components in rabbit serum (Keller and Sorkin, 1965). Furthermore, evidence was presented that fixation of haemolytic complement does not parallel chemotaxis.

It would be useful to know if such serum factors are also present in species other than the rabbit. The finding that they are widely distributed would indicate that the mechanisms may be of general biological importance. Indeed, most substances known to be chemotactic *in vivo* could exert their effect through a common mechanism involving humoral components of the host. However, it has been claimed that chemotaxis of granulocytes in the presence of staphylococci can occur in the absence of serum (Lotz and Harris, 1956). Therefore the action of various agents known to be chemotactic *in vivo* was reassessed *in vitro* by Boyden's technique in the presence of fresh or inactivated serum and in serum-free medium.

MATERIALS AND METHODS

Preparation of human (aHGG) as well as bovine (aBGG) heat-aggregated γ-globulin, polymorphonuclear leucocytes from rabbits and of fresh rabbit and guinea-pig serum has been described previously (Keller and Sorkin, 1965). Blood of sheep and humans was obtained by venepuncture. Calf and pig blood were obtained from the slaughterhouse and sera were prepared similarly to rabbit serum and stored at −25°.

Culture medium. Gey's solution containing 20 per cent inactivated (30 minutes at 56°) rabbit serum was used for preparing cell suspensions for the experiments presented in Tables 1, 2 and 3. For the experiment in Table 4 the granulocytes were suspended in 2 per cent human serum albumin (HSA) in Gey's solution. The Gey's solution contained 10 mg streptomycin and 10,000 units penicillin per litre. The test solution in the experiments of Tables 1, 2 and 3 consisted again of Gey's solution containing 20 per cent serum of various species, as indicated in the tables. Details of the incubation procedures are given in each table. The test mixtures in Table 4 contained no serum. The test agent was diluted with Gey's solution. Prior to incubation in the Boyden chambers the cell suspensions and test solutions were adjusted to pH 7·2.

Rabbit anti-HSA serum. Rabbits were given a series of eight intravenous injections of 1 ml normal human serum over a period of 5 weeks. After a rest period of 5 weeks a booster injection was given and the animals were bled 5 days later. Antisera were inactivated for 30 minutes at 56° before use.

Antigen–antibody complexes (HSA–anti-HSA). Precipitates were prepared in the equivalence zone. In experiments with human serum, HSA was not added and precipitates were prepared in the region of antigen excess.

Human serum albumin (HSA). For preparation of antigen–antibody complexes, HSA obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service, Bern, was employed at a concentration of 1 mg/ml in Gey's solution.

Tuberculin (PPD) (The State Serum Institute, Copenhagen). A solution was prepared in physiological saline at a concentration of 2 mg/ml.

Human heat-aggregated γ-globulin (aHGG). The stock solution used for the experiments described in Table 1 contained 40 mg/ml in Gey's solution and in Table 3, 19 mg/ml in Gey's solution.

Bovine heat-aggregated γ-globulin (aBGG). The concentration of the stock solution was 40 mg/ml in Gey's solution.

Glycogen (Fluka AG, Buchs, Switzerland). A solution was prepared at a concentration of 30 mg/ml in physiological saline.

Witte's peptone. The preparation used was kindly supplied by Dr R. Keller, Zürich. It was dissolved in physiological saline at a concentration of 10 mg/ml.

Bacto-Casitone (Difco). The stock solution contained 10 mg/ml physiological saline.

Casein. The preparation used was made according to Hammersten (Merck, Darmstadt, Germany). The stock solution contained 10 mg/ml in physiological saline.

Proteus *endotoxin.* This preparation (kindly supplied by Dr B. Schär, Ciba AG, Basel) was dissolved in physiological saline at a concentration of 1 mg/ml.

All of the above solutions were sterilized by filtration.

Staphylococcus albus. This organism (kindly supplied by Professor E. Wiesmann, Zürich) was grown for 24 hours at 37° in a medium containing 0·2 per cent glucose in physiological saline to avoid contamination with peptone or macromolecular substances. The bacteria were killed by heating the culture three times to 64° for 2 hours. The suspension was centrifuged and the deposited cocci resuspended in 10 ml of sterile physiological saline. The final concentration was 65×10^6 cocci per ml saline.

Measurement of chemotaxis. The method has been used as described by Boyden (1962), except that the chambers were incubated in air and not in 5 per cent carbon dioxide. Each chamber contained $5–7 \times 10^6$ granulocytes.

RESULTS

INDUCTION OF CHEMOTAXIS BY SERUM OF VARIOUS MAMMALIAN SPECIES AFTER INCUBATION WITH AGGREGATED γ-GLOBULIN OR ANTIGEN–ANTIBODY MIXTURES

Sera of animals of different species were incubated with bovine heat-aggregated γ-globulin, human heat-aggregated γ-globulin or antigen–antibody complexes and their chemotactic effects were evaluated. The results are presented in Table 1. These data were obtained in experiments performed on different days and cells from different animals were used with each serum. Consequently, only data from the same species should be compared.

TABLE 1

THE PRODUCTION OF CHEMOTACTIC EFFECTS IN THE SERUM OF VARIOUS MAMMALIAN SPECIES UPON INCUBATION WITH AGGREGATED γ-GLOBULINS OR ANTIGEN–ANTIBODY MIXTURES

Origin of serum	Cells/field			
	(A) Control	(B) aHGG	(C) aBGG	(D) HSA–anti-HSA
Rabbit	13	82	133	305
Guinea-pig	6	10	15	474
Pig	7	12	17	248
Calf	0	5	3	238
Sheep	2	1	1	140
Man	0	1	1	39*

The test mixtures were prepared as follows:

A. 0·6 ml serum—5 minutes at 37°—5 minutes at 37°; 30 minutes at 56°—2·4 ml Gey's solution.

B. 0·6 ml serum—5 minutes at 37°—0·03 ml aHGG (40 mg/ml)—5 minutes at 37°; 30 minutes at 56°—2·4 ml Gey's solution.

C. 0·6 ml serum—5 minutes at 37°—0·03 ml aBGG (40 mg/ml)—5 minutes at 37°; 30 minutes at 56°—2·4 ml Gey's solution.

D. 0·4 ml serum—5 minutes at 37°—0·2 ml antiserum (inactivated)—0·2 ml HSA (1 mg/ml) —5 minutes at 37°; 30 minutes at 56°—2·2 ml Gey's solution.

* For assay in human serum 0·2 ml HSA (1 mg/ml) was replaced by 0·2 ml of Gey's solution.

Antigen–antibody complexes were found to exert a pronounced chemotactic effect upon incubation with each of the sera tested. Heat-aggregated bovine γ-globulin and heat-aggregated human γ-globulin were chemotactic only in rabbit serum. No significant activity was detected when these γ-globulins were incubated in fresh serum of guinea-pig, pig, calf, sheep or man.

EFFECT OF HEAT-INACTIVATION OF VARIOUS SERA ON THE PRODUCTION OF CHEMOTACTIC ACTIVITY ON INCUBATION WITH ANTIGEN–ANTIBODY MIXTURES

When the serum was heated for 30 minutes at 56° before the addition of antigen–antibody complexes the formation of chemotactic mediator(s) was prevented. When the mixtures were heated for 30 minutes at 56° after incubation of antigen–antibody complexes in fresh serum their chemotactic effect was not impaired. Hence, the newly generated chemotactic factor(s) were heat-stable.

This experiment was done with sera from rabbit, guinea-pig, sheep and humans. The results are given in Table 2. For the same reasons as given for Table 1, the data are only comparable within the species.

TABLE 2

EFFECT OF HEAT-INACTIVATION OF VARIOUS SERA ON THE PRODUCTION OF CHEMOTACTIC ACTIVITY ON INCUBATION WITH ANTIGEN–ANTIBODY MIXTURES

Origin of serum	Cells/field		
	(A) Control	(B) Fresh serum +Ag/Ab	(C) Inactivated serum +Ag/Ab
Rabbit	3	381	6
Guinea-pig	1	330	20
Pig	2	279	5
Calf	0	238	12
Sheep	14	283	31
Man	0	66*	4*

The test mixtures were prepared as follows:

A. 0·6 ml serum—5 minutes at 37°—5 minutes at 37°—30 minutes at 56°—2·4 ml Gey's solution.

B. 0·4 ml serum—5 minutes at 37°—0·2 ml antiserum (inactivated)—0·2 ml HSA (1 mg/ml)—5 minutes at 37°—30 minutes at 56°—2·2 ml Gey's solution.

C. 0·4 ml serum—5 minutes at 37°—0·2 ml antiserum (inactivated)—5 minutes at 37°—30 minutes at 56°—0·2 ml HSA (1 mg/ml)—2·2 ml Gey's solution.

* For assay in human serum 0·2 ml HSA (1 mg/ml) was replaced by 0·2 ml of Gey's solution.

TABLE 3

CHEMOTACTIC EFFECT OF VARIOUS AGENTS IN THE PRESENCE OF FRESH OR HEAT-INACTIVATED NORMAL RABBIT SERUM

Agents incubated in normal rabbit serum	Final concentration in serum	Cells/field		
		(A) Control, no agent	(B) Fresh serum +agent	(C) Inactivated serum+agent
HSA–rabbit-anti-HSA	See Table 2	3	381	6
Heat-aggregated human γ-globulin	2 mg/ml	1	369	1
PPD	0·26 mg/ml	3	106	3
Glycogen	10 mg/ml	1	60	5
Proteus endotoxin	0·01 mg/ml	2	327	3
Staph. albus (heat-killed)	13×10^6/ml	2	71	3
Witte's peptone	1 mg/ml	3	45	35

The test solutions, final volume 3 ml, were prepared as follows:

A. 0·6 ml serum—5 minutes at 37°—5 minutes at 37°—30 minutes at 56°—2·4 ml Gey's solution.

B. 0·6 ml serum—5 minutes at 37°—*x*ml of agent stock solution—5 minutes at 37° —30 minutes at 56°—Gey's solution ad 3·0 ml.

C. 0·6 ml serum—5 minutes at 37°—5 minutes at 37°—30 minutes at 56°—*x*ml of agent stock solution—Gey's solution ad 3·0 ml.

Volume *x* of agent stock solution used: Heat-aggregated human γ-globulin 0·063 ml; PPD 0·1 ml; glycogen 0·36 ml; *Proteus* endotoxin 0·006 ml; *Staph. albus* 0·15 ml; Witte's peptone 0·066 ml.

From the results of Table 2, it can be seen that all sera, when incubated with antigen–antibody complexes, exert their chemotactic activity only in the presence of fresh serum containing heat-labile components.

EFFECT OF HEAT-INACTIVATION OF RABBIT SERUM ON THE CHEMOTACTIC EFFECT OF VARIOUS AGENTS

The experiments which follow evaluate the significance of these serum mechanisms for the chemotactic activity of agents other than antigen–antibody complexes or heat-aggregated human γ-globulin. From the many agents known to induce leucocyte accumulation *in vivo* PPD, glycogen, *Proteus* endotoxin, *Staph. albus* and Witte's peptone were selected for tests *in vitro*. The results given in Table 3 show that PPD, glycogen, *Proteus* endotoxin and heat-killed *Staph. albus* exerted their chemotactic effect only in the presence of fresh serum containing heat-labile components.

Since, for practical reasons these agents had to be tested separately, the data do not allow a quantitative comparison between these agents.

THE CHEMOTACTIC EFFECT OF VARIOUS AGENTS IN THE ABSENCE OF SERUM

The question whether chemotaxis could occur in the total absence of serum was tested in the following way. The same amounts of the various chemotactic agents described in Table 3 were dissolved in Gey's solution alone. Their chemotactic activity on rabbit granulocytes suspended in 2 per cent HSA in Gey's solution is shown in Table 4.

TABLE 4
THE CHEMOTACTIC EFFECT OF VARIOUS AGENTS ON RABBIT GRANULOCYTES IN THE ABSENCE OF SERUM

Agent in Gey's solution	Cells/field
None	0
HSA–rabbit-anti-HSA	0
Heat-aggregated human γ-globulin (0·2 mg/ml)	0
Heat-aggregated bovine γ-globulin (0·2 mg/ml)	0
PPD (0·066 mg/ml)	2
Glycogen (3·6 mg/ml)	0
Proteus endotoxin (2 μg/ml)	0
Staph. albus ($3{\cdot}2 \times 10^6$/ml) (heat-killed)	4
Witte's peptone (220 μg/ml)	156
Bacto-Casitone (2 mg/ml)	126
Casein (Hammersten) (2 mg/ml)	240

For evaluation of chemotaxis the granulocytes were suspended in Gey's solution containing 2 per cent HSA.

The results show that antigen–antibody complexes, heat-aggregated human γ-globulin, heat-aggregated bovine γ-globulin, glycogen, PPD, *Proteus* endotoxin and heat-killed *Staph. albus* exert no chemotactic effect in the absence of fresh serum. Contrariwise Witte's peptone, Bacto-Casitone and casein (Hammersten) showed distinct chemotactic activity under the same conditions.

DISCUSSION

The results obtained in the present work confirm the findings of Boyden (1962) that antigen–antibody complexes exert their chemotactic effect on rabbit granulocytes by formation of heat-stable chemotactic factor(s) from rabbit serum containing heat-labile components. They show in addition that fresh sera of guinea-pig, calf, sheep and humans also contain similar heat-labile factors productive of heat-stable factor(s) which are chemotactic for rabbit granulocytes. This is of special interest since it points to the generality of the phenomenon. Tests with cells from species other than the rabbit are however necessary to substantiate such a conclusion. The reason for the low activity of human serum incubated with rabbit anti-HSA serum can best be explained by the large excess of the antigen (HSA). Boyden (1962) found that chemotactic activity was maximal for complexes prepared in the equivalence zone, and decreased markedly with antigen excess.

As indicated in Table 1, human as well as bovine aggregated γ-globulins are chemotactic provided they are incubated in rabbit serum. However, these aggregated γ-globulins have no significant effect when interacted with sera of guinea-pig, pig, calf, sheep, and humans. At the present time these differences remain unexplained.

A previous study (Keller and Sorkin, 1965) showed that human heat-aggregated γ-globulin fixed haemolytic complement of the guinea-pig, but did not produce a chemotactic effect upon incubation in guinea-pig serum. On the other hand, antigen–antibody complexes had a pronounced chemotactic as well as complement fixing activity. It has been shown that human heat-aggregated γ-globulin also fixes pig, sheep and human haemolytic complement (R. Keller, unpublished experiments). Nevertheless, its incubation with these sera produced no chemotactic effect (Table 1). Thus these results are in harmony with our previous findings of lack of parallelism between formation of chemotactic factor(s) and fixation of haemolytic complement.

As shown in Table 3 incubation of PPD, glycogen, *Proteus* endotoxin or heat-killed *Staph. albus* with fresh rabbit serum containing heat-labile components led to the formation of heat-stable chemotactic factor(s). Thus as observed with antigen–antibody complexes or human heat-aggregated γ-globulin heat-labile serum factors were found to be necessary for chemotaxis. These results are compatible with the hypothesis that most agents that show the property of inducing migration of leucocytes *in vivo* also exert their effect by a common serum mechanism. Further studies will be necessary to determine whether the mechanism is identical for all these agents and for all sera. It may be that PPD, *Proteus* endotoxin and heat-killed *Staph. albus* induce chemotaxis by formation of complexes with their 'natural antibodies' present in the normal rabbit serum.

The high concentration of glycogen necessary to induce chemotaxis may be an indication that some impurity such as endotoxin rather than glycogen itself is responsible for the chemotactic effect.

The concept that different agents may exert their chemotactic effects by generating common mediator(s) differs from earlier views (McCutcheon, 1946; Lotz and Harris, 1956). According to these authors chemotactic agents released from bacteria for example would act directly on the cells.

Our results in the present test system obtained with *Staph. albus* differ also fundamentally from those obtained by Lotz and Harris (1956). These authors found that human polymorphonuclear leucocytes are attracted by *Staph. albus* in the absence of serum. It is

unlikely that the use of human instead of rabbit cells can explain these differences. It is also improbable that serum components on the surface of the granulocytes were involved in the chemotactic effect of staphylococci. An alternative explanation could be that the bacteria were contaminated by a chemotaxis-producing factor present in the bacteriological culture medium. This appears possible since Witte's peptone and Bacto-Casitone, which are widely used in bacteriological media, were found to be chemotactic in our serum-free test system (Table 4).

The chemotactic activity of Witte's peptone in the serum-free test system was even more pronounced than in media containing fresh or inactivated serum. Therefore it appears improbable that serum components on the surface of the cells are involved in this effect. The possibility that Witte's peptone contains substances structurally related to chemotactic factor(s) has therefore to be considered.

Similar to the pancreatic casein digest (Bacto-Casitone), casein itself had a pronounced chemotactic effect in serum-free medium. The casein used was prepared according to the method of Hammersten, which involves repeated precipitation at acid and alkaline pH. This treatment may generate hydrolytic products. Thus this finding is compatible with the idea expressed by earlier workers that degradation of proteins may lead to formation of chemotactic substances (Menkin, 1940). It is noteworthy however, that peptides such as bradikinin or kallidin were not chemotactic in our serum-free test system in concentrations from 10^{-5} to 10^{-9}. Glycogen and peptone are commonly used for production of exudates and there is at present no evidence that both activate the granulocytes in the same manner. This may be an important consideration in the choice of the agent for induction of peritoneal exudates. The present experiments were performed with cells from peritoneal exudates induced by glycogen. It was an interesting finding that these cells are still capable of responding to the same stimulus *in vitro*, while in the controls no migration occurred.

ACKNOWLEDGMENTS

The authors are indebted to Mr H. Hoch for technical assistance. This work was supported by the Swiss National Foundation for Scientific Research, Grant No. 2635 and by the World Health Organization.

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