

## TISSUE CULTURE STUDIES ON BACTERIAL HYPERSENSITIVITY

### IV. PROTECTIVE EFFECT OF IMMUNE PLASMA AGAINST THE DELETERIOUS INFLUENCE OF STREPTOCOCCAL EXTRACT ON HYPERSENSITIVE CELLS

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(Received for publication, January 29, 1937)

Crude hemolytic streptococcal extract in proper concentration exerts a comparatively greater toxic action on cells from guinea pigs infected with these microorganisms than on normal cells when studied by the tissue culture technique (1). That this action is relatively specific was established by comparing it with that of various other bacterial extracts and products; only those prepared from homologous strains or from strains in the same serological group C (2) exerted a specific inhibiting influence on the sensitive cells. It is not improbable that a guinea pig elaborates similar toxic substances in foci chronically infected with these microorganisms, yet in spite of persisting cellular sensitivity (1) during the chronic stage of the infection, the animals appear healthy, gain weight, and seem relatively unaffected by the large abscesses containing many living streptococci. This phenomenon might be explained by the presence in the blood of a factor capable of protecting the sensitive cells of infected animals from these hypothetical toxic substances; and if such a factor exists there, it might be subject to detection and analysis in tissue cultures.

The present communication reports experiments which test the validity of the foregoing hypothesis. Because agglutinins and precipitins had been detected throughout the course of this chronic streptococcal infection (1), the association of these antibodies with the hypothetical protective substance was also studied.

## EXPERIMENTAL

Guinea pigs were infected with a group C (Lancefield) hemolytic streptococcus, strain K 104. The experimental methods, tissue culture media, type of observations, and quantitative formulae are similar to those detailed previously (1, 3). The plasmas from normal and infected animals are designated normal and immune, respectively. Comparative cytotoxic indices of bacterial extract for sensitive cells from infected animals when these cells were grown in normal plasma, with and without bacterial extract, and when grown in immune plasma, with and without bacterial extract, afford data for the analysis of the possible protective action of immune plasma. Thus in a typical experiment, 8 different conditions were imposed, and using 12 explants for each condition, a total of 96 was required. The rates of growth were determined in the following 8 experimental conditions.

1. Sensitive cells grown in normal plasma containing bacterial extract.<sup>1</sup> (Snb)<sup>2</sup>
2. Normal " " " " " " " " " " (Nnb)
3. Sensitive cells grown in normal plasma. (Sn)
4. Normal " " " " " " " " " " (Nn)
5. Sensitive cells grown in immune plasma containing bacterial extract. (Sib)
6. Normal " " " " " " " " " " (Nib)
7. Sensitive cells grown in immune plasma. (Si)
8. Normal " " " " " " " " " " (Ni)

The comparative cytotoxic index of bacterial extract for sensitive cells grown in normal media was determined by quantitative measurements of cellular migration in the first 4 experimental conditions, and is expressed by Formula 1 =  $\frac{\frac{Snb}{Sn}}{\frac{Nnb}{Nn}}$

The method for determining this index has been fully described (1, 3). Likewise, the comparative cytotoxic index of bacterial extract for sensitive cells grown in immune plasma was calculated by quantitative measurements of cellular migration in the 5th to 8th experimental conditions and is expressed in Formula

$$2 = \frac{\frac{Sib}{Si}}{\frac{Nib}{Ni}}$$

<sup>1</sup> The final concentration of bacterial extract in the media was 1 to 6,000, a concentration which had only slight inhibitory effect on normal cells.

<sup>2</sup> The formulae express by means of letters the set up of each experimental condition. The capital letters "S" and "N" indicate sensitive and normal cells, respectively. The small letters "n" and "i" indicate normal and immune plasmas, respectively, while "b" indicates bacterial extract. For example: Snb shows that sensitive cells were grown in normal media containing bacterial extract, etc.

Obviously, if  $\frac{\frac{S_{nb}}{N_{nb}}}{N_n} = \frac{\frac{S_{ib}}{N_{ib}}}{N_i}$  the value, or effect, of n (normal plasma) and

i (immune plasma) must be equivalent, because the other factors in the formulae are identical; hence there would have been no neutralization of the toxic effect of the bacterial extract b on the sensitive cells S. When, on the other hand, the value of Formula 2 is greater than that of 1, the factor responsible for this difference must be accounted for by the difference between the normal plasma n and the immune plasma i; and the quantitative difference in the two comparative indices is an approximate expression of the relative protective action of the immune plasma i. The greater the value of Formula 2 over that of Formula 1, the proportionately greater is the neutralizing effect of the immune plasma. Microscopic appearances of the cells under the various experimental conditions also afford qualitative evidence of cellular injury, which can be correlated with the quantitative data.

#### RESULTS

In six experiments there was tested the capacity of immune serum to neutralize the toxic action of streptococcal extract on sensitive splenic cells, obtained from guinea pigs which had been infected for from 4 to 20 weeks. The degree of sensitivity of the cells grown in normal plasma as expressed by the comparative cytotoxic indices (Table I, column 8), is in inverse proportion to the index, which varied from 0.35 to 0.90.

When the same tissues, sensitive and normal, were tested with bacterial extract combined with immune plasma, the toxic action of the streptococcal extract was definitely less, as is indicated by the comparative cytotoxic indices which varied from 0.57 to 1.14 (Table I, column 9). Two of the indices (Experiments 213 and 223) were 1.12 and 1.14, respectively, which demonstrates complete neutralization of the specific toxic effect of the streptococcal extract by the two immune plasmas, respectively. In the other experiments a comparison of the two sets of indices indicates only partial neutralization by the immune plasmas. Qualitatively the microscopic appearances of the cells in the various flasks confirmed the quantitative data: the sensitive cells grown in a mixture of bacterial extract and immune plasma had a much healthier appearance than those grown with similar extracts and normal plasma. The plasmas in all experiments

except Nos. 213 and 253 were derived from infected and normal animals other than those from which the splenic explants were obtained.

Similar experiments were performed with plasmas from animals with acute infections of only 7 and 10 days' duration, a period before precipitins and increased agglutinin titers were demonstrable. These plasmas, however, were so intrinsically toxic that even without the addition of bacterial extract they greatly inhibited cellular activity of normal explants; hence it was impossible to test their capacity to neutralize the toxic effect of streptococcal extract on sensitive cells.

TABLE I

Column (1)	(2)	(3)	(4)		(5)		(6)	(7)	(8)	(9)	(10)		
			Antibody titer of normal plasma (serum) (n)		Antibody titer of immune plasma (serum) (i)							Compara- tive cytotoxic index of bacterial extract in normal plasma Sab $\frac{S_n}{Nnb}$ $\frac{Nn}{Nn}$	Compara- tive cytotoxic index of bacterial extract in immune plasma Sib $\frac{S_i}{Nib}$ $\frac{Ni}{Ni}$
			Agglu- tinin	Precip- itin	Agglu- tinin	Precip- itin							
Experiment No.	Duration of infection	Duration of infection											
	<i>wks.</i>	<i>wks.</i>											
213	10	10	40	—	320	+	0.90	1.12	Complete				
219	7	9	Not done	—	Not done	+	0.48	0.57	Partial				
223	4	4	40	—	640	+	0.69	1.14	Complete				
235	6	6	160	—	320	+	0.55	0.73	Partial				
253	21	20	20	—	1,280	+	0.35	0.68	"				
254	14	14	10	—	640	+	0.42	0.67	"				

*Antibody Content of Normal and Immune Sera.*—Agglutinin titers and precipitin reactions, determined as previously described (1), are shown in Table I. None of the normal sera and all of the immune sera contained precipitins. The agglutinin titers of the normal sera varied from 10 to 160 and of the immune sera from 320 to 1280. The normal serum with an agglutinin titer of 160 is abnormally high since the average titer for 21 normals (1) was found to be 43. Possibly this one high titer was due to a previous spontaneous infection with hemolytic streptococci, a type of infection very common among ordinary stocks of these animals.

*Correlation between Agglutinin Titers and Neutralizing Capacities of Immune Plasmas.*—The small number of determinations makes generalizations concerning such correlation difficult, and because the degree of sensitivity of the cells to bacterial extract in normal plasma varied in different experiments, comparison of the quantitative neutralizing capacity of different immune plasmas must be relative. The relationship, called index of relative neutralizing capacity of immune plasma, is expressed numerically by dividing the value of Formula 2 by that of Formula 1.

Table II shows that the index varied between 1.18 and 1.94, with the higher values indicative of greater neutralizing power. When

TABLE II  
*Correlation between Agglutinin Titers and Neutralizing Capacities of Immune Plasmas*

Experiment No.	Comparative cytotoxic index of bacterial extract in normal plasma (Formula 1)	Comparative cytotoxic index of bacterial extract in immune plasma (Formula 2)	Index of relative neutralizing capacity of immune plasma (Formula 2) (Formula 1)	Agglutinin titer of immune plasma (serum)
213	0.90	1.12	1.24	320
219	0.48	0.57	1.18	Not done
223	0.69	1.14	1.65	640
235	0.55	0.73	1.33	320
253	0.35	0.68	1.94	1,280
254	0.42	0.67	1.60	640

these indices are compared with the agglutinin titers of the respective sera it is evident that there is a rough but definite correlation between the agglutinating and neutralizing capacities of corresponding bloods.

In order to demonstrate that the neutralizing capacities of the immune plasmas were not due to variable non-specific effects of the different plasmas on sensitive and normal cells, the initial growth energies of the sensitive and normal tissues were compared when grown in normal and immune plasmas in the absence of bacterial extract. The comparative initial growth energy index =

$$\frac{\text{Rate of growth of test explants in normal or immune plasma}}{\text{" " " " normal " " " " " "}} \text{ or } \frac{S_n}{N_n} \text{ and } \frac{S_i}{N_i} \text{ respectively.}$$

In Table III these comparative values are recorded for each of the six experiments. The close correspondence of the respective values in the two columns of indices shows that the plasmas themselves did not exert either a selective toxic or stimulating effect on either the normal or sensitive tissues. These data strengthen the conception that some factor in the immune plasma neutralizes a component of the bacterial extract that is toxic for sensitive cells.

*Failure to Neutralize with Immune Serum the Skin Reacting Substances in Bacterial Extract.*—Various combinations of bacterial extract and immune serums containing both precipitins and agglutinins were tested on streptococcal infected animals showing cutaneous hyper-reactivity to bacterial extract. Some of the combinations of bacterial

TABLE III  
*Comparative Initial Growth Indices in Normal and Immune Plasmas*

Experiment No.	Comparative initial growth index in normal plasma $\frac{S_n}{N_n}$	Comparative initial growth index in immune plasma $\frac{S_i}{N_i}$
213	0.91	0.96
219	1.65	1.62
223	1.31	1.44
235	0.68	0.67
253	0.96	0.91
254	1.13	1.12

extract and immune serum were injected soon after mixing, others after incubating for 2 hours, still others after incubating for 2 hours, storing overnight in the ice box, and removing the precipitate. In no test was there evidence that the substance inducing skin reactions was neutralized by the immune sera. This suggests either that different substances in the extracts are responsible for eliciting cutaneous reactions and for injuring cells in tissue cultures, or that the mechanism of responses are different. It is even possible that a mixture may have to be quite exactly balanced for neutralization to be demonstrable *in vivo*.

#### DISCUSSION

A guinea pig inoculated with some strains of group C hemolytic streptococci passes through several stages that have certain analogies

to those seen in some human infections. For a period of about 2 weeks after inoculation the animal appears acutely ill, has fever, and loses weight, and during the first part of the infection there are marked local signs of inflammation at the site of inoculation. Then the local lesion breaks down, discharges its contents, and heals; but the satellite lymph nodes and those more distant become the sites of chronic lesions in which large numbers of streptococci are harbored. The animal develops hypersensitivity to products of these streptococci, hypersensitivity that can be demonstrated in the skin, and at the same time *in vitro*, since sensitive mesenchymal cells are specifically injured in tissue cultures containing extracts of the streptococci. After the 2nd week, however, even though the animal's tissues are still sensitive to streptococci which are growing in large numbers in its body, the guinea pig thrives, is fever-free, and shows no obvious general toxic manifestations. Whatever may be the mechanism that protects the animal, this mechanism obviously neither eliminates the irritating streptococci nor renders the cells less susceptible to the streptococcal toxic products. It seemed of more than passing interest that coincidentally with the appearance of circulating antibodies in the blood stream the animal's general condition improves; and this suggested that some humoral substance might protect the sensitive cells from toxic factors elaborated in the areas of chronic focal infection.

As a matter of fact, the present study shows that plasmas from animals infected with group C streptococci do neutralize the factor in streptococcal extracts which is responsible for the toxic action on sensitive cells in tissue cultures; moreover, this neutralizing effect is roughly quantitatively parallel with the concentration of agglutinins in the respective sera. It is as yet impossible to tell the nature of this neutralizing substance, for the method of demonstrating it is different from those usually employed for detecting antibodies. As these susceptibilities, toxic components, and neutralizing factors are all present in the animal body, it would appear that we have a mechanism whereby the animal's tissues are protected even in the presence of a toxic agent, and hence the chronic infection is well tolerated. If, on the other hand, the neutralizing factor is insufficient in amount to render the toxic substances entirely harmless, this condition of affairs may account for a continuing effect of the focal infection on distantly

situated sensitive cells. Since our experimental animals have been usually efficient in producing this neutralizing factor, few opportunities for studying this phase of infection have presented themselves. Experiments are in progress on inhibition of antibody formation which may throw further light on this problem.

#### SUMMARY

1. Plasmas from guinea pigs, chronically infected with group C hemolytic streptococci, neutralize the components of bacterial extract which exert a marked toxic action on hypersensitive cells *in vitro*.

2. The neutralizing capacity of these immune plasmas is relatively specific for the bacterial extract, and is not due to a variable non-specific effect on normal or hypersensitive tissue cells.

3. A rough correlation between the agglutinin titer and the relative neutralizing capacity of immune plasma suggests that the latter may be a manifestation of antibody action.

4. The tolerance by guinea pigs of chronic hemolytic streptococcal lymphadenitis is explainable, at least in part, by the neutralizing capacity of their plasmas, since such soluble bacterial products as may be absorbed from infectious foci would probably be neutralized before they could exert a deleterious influence on the hypersensitive cells of the animals.

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