J. Hyg., Camb. (1960), **58**, 35
Printed in Great Britain

Heat labile and heat stable factors and antibodies in normal and immune sera against T-system bacteriophages

By ENVER TALI ÇETIN
*The London School of Hygiene and Tropical Medicine**

(*Received* 2 *September* 1959)

INTRODUCTION

Normal serum of guinea-pig and rabbit inactivates T-system coli phages to different extents. This may be due to antibodies that are present in normal serum or to some other factors. Previously, normal serum had been thought to contain antibodies against bacteriophages (Hershey, Kalmanson & Bronfenbrenner, 1943). Jerne demonstrated a specific antibody A, not a neutralizing antibody, against T_4-bacteriophage in normal horse serum (Jerne, 1956). Hershey & Bronfenbrenner (1947) claimed that complement had some effect on neutralization of bacteriophage by serum. The inactivating effect of animal serum against bacteriophage rises after a few injections of homologous bacteriophage. Normal or immune sera lose some of this inactivating power when they are heated at 58° C. for 60 min.

The present paper reports that, in normal guinea-pig and rabbit sera which inactivate phages of the T-system, there are factors of two kinds, one heat labile and the other heat stable. There are heat labile and heat stable antibodies against T_2, T_3 and T_4 phages in immune guinea-pig sera. Complement does not affect the inactivation or neutralization of T_2, T_3, T_4 phages by normal or immune guinea-pig sera.

MATERIALS AND METHODS

Stock lysates of the T-system phages, that were used in the experiments and in the immunization of animals, were prepared in broth with *Escherichia coli* strain B as host. The titres of the lysates were 1×10^{10} to 3×10^{10} per ml. To prepare immune sera, 1 ml. phage stock was injected subcutaneously into guinea-pigs twice a week, 8 ml. of blood being taken by heart puncture before every injection. The sera were designated by the numbers of injections given to produce them. For instance: serum IV was taken after four injections. For each batch of complement, ten guinea-pigs were bled from the throat and their sera were pooled. A part of each serum was heated at 58° C. for 60 min., all sera and complements were kept at $-70°$ C. in a CO_2 ice box, and their complement titres, measured with a red cell indicator system, were determined on the same day for each experiment. In order to determine the haemolytic titres of the sera of guinea-pigs, a suspension of 0·5 % sheep red cells was prepared, and mixed with the same volume of haemolytic serum (diluted to haemolytic titre); 0·2 ml. of sensitized red cells were

* Present address: Dr E. T. Çetin, Assistant Professor, Tip Fakültesi, Mikrobiyoloji Enstitüsü, Universite Istanbul, Turkey.

placed in each tube, and 0·05, 0·1, 0·125, 0·150, 0·175 and 0·200 ml. of serum diluted 1/200 were added to different tubes. In order to determine the haemolytic titres of the sera of rabbits, after putting 0·2 ml. of sensitized red cells in a series of tubes, 0·05, 0·1, 0·150, 0·175, 0·200, 0·250 ml. of serum diluted 1/10 was respectively added to each tube. The contents of each tube were made up to 0·8 ml. with saline. The tubes were incubated for 30 min. in a water-bath at 37° C. and the results were recorded.

All dilutions of phages and sera were made in broth (20 g. Bacto-peptone, 3 g. Bacto beef extract, 5 g. NaCl, 1 g. glucose, 1000 ml. dist. H_2O, pH 7·2). The solid medium used consisted of 11·5 g. Bacto agar, 10 g. Bacto-tryptone, 8 g. NaCl, 1000 ml. dist. H_2O (pH 7·2).

Active phage was assayed by the agar layer method (Gratia, 1936).

EXPERIMENTS

The inactivating power of normal guinea-pig and rabbit sera against T-system coli phages

Heated and unheated normal guinea-pig and rabbit sera inactivate T-system phages to different extents (Figs. 1, 2). In these experiments phage stocks were diluted to contain 5×10^6 phage particles per ml., mixed with the same volume of serum and put in a 37° C. water-bath for 3 hr. The phages were usually stable in broth at 37° C. for 3 hr. The sera were thus diluted 1/2 in these experiments. Inactivation was greatest with undiluted serum; it decreased rapidly with dilution.

Normal guinea-pig serum was most active against T_2 and T_3 phages. It was less active against T_4 and T_6 phages, which are serologically related to T_2 phage, but more active against T_4 phage, which is more closely related to T_2 phage (Delbrück, 1946), than against T_6 phage. The normal serum lost some inactivating power when heated at 58° C. for 60 min. The inactivation of T_2 phage by exposure for 3 hr. to different guinea-pig sera varied between 68 and 90 % with heated sera and 90 and 99 % with unheated sera.

Normal rabbit serum was most active against T_3 phage. Among the T-even phages, it was most active against T_6 phage. It was more active against T_2 phage which is more closely related to T_6, than against T_4 phage. Normal rabbit serum also lost some of this inactivating power when heated at 58° C. for 60 min. (Fig. 2).

Neutralizing effect of immune sera

In these experiments also, phage stocks were diluted to contain 5×10^6 phage particles per ml. and after mixing with the same volume of guinea-pig serum were placed in a water-bath at 37° C. for 30–120 min. according to the serial number of the serum. The inactivating power of heated and unheated sera rose within a week after injection of phage and increased further after more injections (Fig. 3). As described below, there was no change in the neutralizing ability of heated or unheated sera, when normal guinea-pig serum was added as complement, if allowance was made for the normal inactivating power of the added serum or if this was absorbed out in the cold with homologous phage. Both heat labile and heat

stable antibodies were present in sera after injections of phage and increased after more injections. This result suggests that both heat labile and heat stable antibodies are produced against the phage injected.

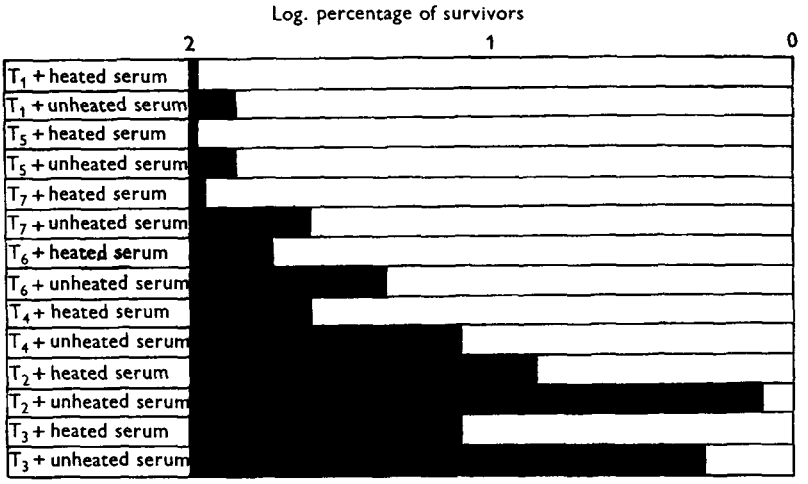

Fig. 1. Inactivation of T phages by exposure for 3 hr. to heated and unheated pooled normal guinea-pig sera at final dilution 1/2. (This and each of the following histograms is based on the results of numerous experiments, and is so arranged that black columns represent the amount of phage inactivated.)

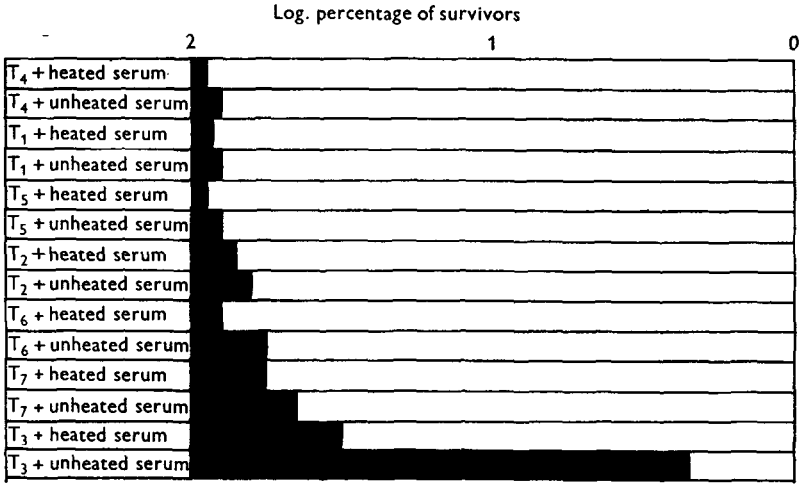

Fig. 2. Inactivation of T phages by exposure for 3 hr. to heated and unheated pooled normal rabbit sera at final dilution 1/2.

Occurrence of partly neutralized phage

After exposure of the phages to normal or immune sera, survivors producing plaques smaller than normal were found in addition to those producing plaques of normal size. Phages from the small plaques gave normal-sized plaques when

https://doi.org/10.1017/S0022172400038110 Published online by Cambridge University Press

replated. Jerne showed that the small plaques, found when T_4 phage was exposed to antiserum taken early in the course of immunization, were due to reactivation of neutralized phage particles (Jerne & Avegno, 1956). In other cases, they have been attributed to partly neutralized phages (Andrewes & Elford, 1933) and Dr Elinor Meynell (personal communication).

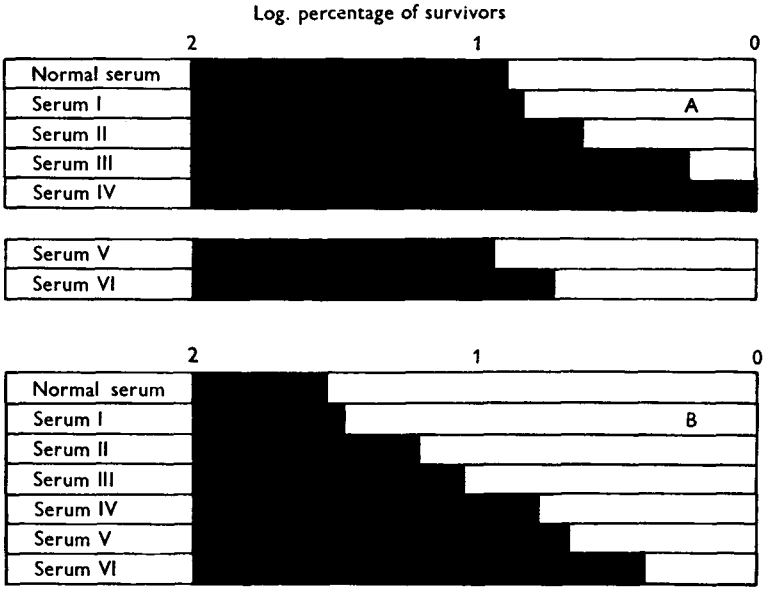

Fig. 3. Inactivation of T_2 phage by (A) unheated and (B) heated normal guinea-pig sera and immune sera taken after from 1 to 6 immunizing injections. Phage was exposed to unheated sera V and VI for 10 min., to all other sera for 25 min. Final dilution throughout was 1/2, undiluted serum being added to the phage suspension.

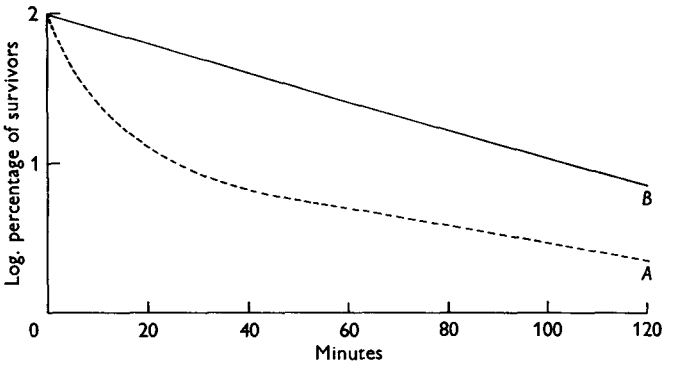

Fig. 4. Inactivation curves of normal guinea-pig serum against T_2 phage. (*A*) Unheated serum; (*B*) heated serum.

Inactivation or neutralization curves with heated and unheated sera

Inactivation of phage by normal unheated serum was fast at first, becoming slower, so that the inactivation curve was concave upwards. The inactivation curve for heated normal serum was nearly a straight line (Fig. 4). The neutraliza-

tion curves for unheated immune sera were again concave upwards, and the more the immunization increased, the more marked did the concavity become. The curves for heated immune sera were nearly straight lines (Fig. 5).

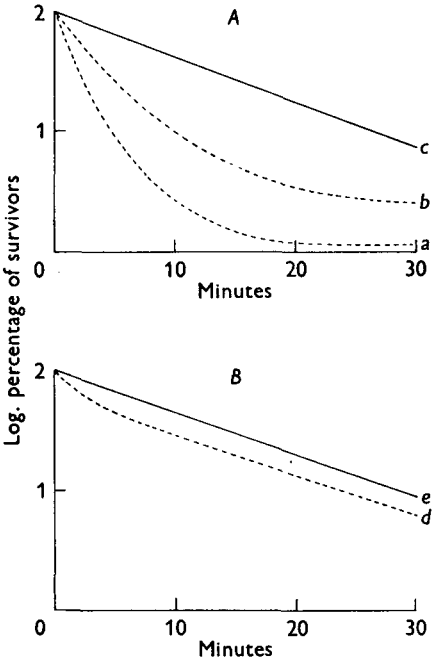

Fig. 5. (*A*) Unheated serum: (*a*) 1·5 ml. serum + 1·5 ml. Ø 10^6; (*b*) 2 ml. *a*, + 0·2 ml. Ø 10^7; (*c*) 1 ml. *b*, + 0·1 ml. Ø 10^7. (*B*) Heated serum: (*d*) 1 ml. heated serum + 1 ml. Ø 10^6; (*e*) 0·5 ml. *d*, + 0·05 ml. Ø 10^7. Neutralization curves of T_2 phage by (*A*) unheated and (*B*) heated anti-T_2 immune guinea-pig serum V.

If, to a mixture of phage with unheated normal or immune serum from early bleedings such that the surviving phage was reduced to 1 %, an equal amount of fresh phage was added in a volume small enough not to alter the dilution of serum significantly (e.g. by adding 0·1 ml. of phage to 1 ml. of the mixture) the inactivation or neutralization curve for the added phage resembled the inactivation or neutralization curve for heated serum; it was a straight line. If, before the second addition of phage, the phage–serum mixture was heated at 58° C. for 60 min., the second neutralization or inactivation curve was not altered. It appeared that the first exposure to phage had depleted the serum of the heat labile factor or antibody, but not of the heat stable, the residue of which was responsible for the effect of the second exposure to phage.

Immune sera from later bleedings contained more heat labile antibodies, and one exposure to phage did not absorb all of them (Fig. 5*b*). The second neutralization curve was not similar or parallel to the neutralization curve of the same serum after heating. Only with a second addition of fresh phage (Fig. 5*c*) did the neutralization curve become similar to that of the same serum heated. These experiments showed that the heat labile activity was exhausted more readily than the heat

https://doi.org/10.1017/S0022172400038110 Published online by Cambridge University Press

stable activity, as is suggested by the shapes of the two kinds of neutralization curve.

Further addition of fresh phage exhausted the heat stable antibodies as well as the heat labile.

When an addition of phage was made to a mixture of phage with heated serum, the second neutralization curve was a straight line similar or parallel to the first (Kalmanson, Hershey & Bronfenbrenner, 1942) (Fig. 5*e*).

Effect of dilution

Normal or immune sera diluted in broth, or immune sera diluted in normal guinea-pig serum, had less inactivating power than the undiluted sera. The heat labile factor was more sensitive to dilution than the heat stable factor: unheated serum lost more of its inactivating power on dilution than did the same serum heated. Figure 6 shows the inactivating and neutralizing effect of normal and immune guinea-pig sera diluted 1/10 in broth. It is easy to observe the greater effect of dilution on the heat labile factor by comparing Figs. 3 and 6.

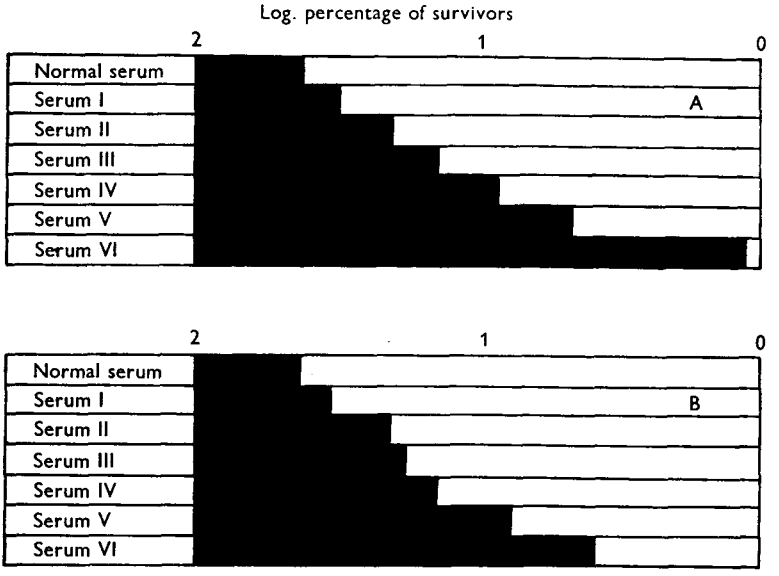

Fig. 6. Inactivation of T_2 phage by (A) unheated, and (B) heated normal guinea-pig sera and immune sera taken after from 1 to 6 immunizing injections. Final dilution of serum 1/10 throughout, all sera being diluted 1/5 with broth before addition of phage suspension. Compare with Fig. 3, which shows the result of similar experiments with undiluted sera.

When sera were diluted 1/10, the inactivating effects of heated and unheated normal sera were reduced. The unheated serum lost more activity than the heated serum and showed the same inactivating effect as the heated serum. When heated and unheated immune sera II and III were diluted 1/10, the unheated sera remained a little more powerful than the heated ones, but when diluted 1/20 heated and unheated immune sera II and III showed the same inactivating effect. As

https://doi.org/10.1017/S0022172400038110 Published online by Cambridge University Press

immunization proceeded, the dilution, at which a difference between heated and unheated sera could no longer be detected, became progressively greater. For instance, dilution rate is 1/80 for immune serum V.

Varied dilutions of heated serum gave a neutralization curve which was nearly a straight line. The neutralization curve of unheated serum became progressively less concave by dilution, until at sufficient dilution it was nearly the same straight line as that obtained with the same serum heated.

Injection of related and unrelated phages

Heat labile and heat stable antibodies against T_2 phage rose in guinea-pig serum after injection of T_4 phage, which was serologically related to T_2 phage. Injections of T_3 phage did not cause an increase in the amount of heat labile and stable factors against T_2 phage in guinea-pig serum.

Haemolytic titres of complement preparations and normal and immune sera

The haemolytic titre of the pooled complement used in the experiments did not change during the period in which all the experiments were done. There were no significant differences between the haemolytic titres of the sera of normal guinea-pigs and rabbits and the sera of those immunized with phages.

Effect of complement on the inactivation or neutralization of bacteriophage by serum

Normal and immune heated and unheated sera were diluted 1/5 in broth or in normal guinea-pig serum, and were mixed with the same volume of bacteriophage. The normal guinea-pig serum used as complement in these experiments inactivated phage by its normal inactivating factor when diluted about 1/2. If allowance was made for this inactivating effect of the normal guinea-pig serum used as complement, immune sera diluted 1/5 showed the same inactivating effect on phage whether diluted in broth or in complement. The addition of complement diluted 1/4 or 1/10 to immune serum did not therefore affect its neutralizing power.

The phage inactivating factor could be absorbed from normal guinea-pig serum by homologous phage. For this adsorption experiment 40 ml. of 2×10^{10} per ml. T_2 phage stock was spun in a Spinco centrifuge at 18,000 r.p.m. for 60 min. The pellet was mixed with 7 ml. of normal unheated guinea-pig serum and the tube was kept in crushed ice in the cold room for 60 min. It was then spun again at 18,000 r.p.m. for 90 min. and the supernatant was taken. The haemolytic titres of normal guinea-pig serum and of the supernatant were the same, indicating that no complement had been lost during the absorption. The supernatant mixed with T_2 phage produced no inactivation. The phage inactivating factor had thus been removed without changing the complement titre of the serum, as judged by haemolytic activity.

The supernatant was added to mixtures of serum and bacteriophage to find out its effect on neutralization. The final dilutions of the supernatant in the mixtures were 1/2, 1/4 and 1/10. The results were the same as those controls without the

complement-containing supernatant. These experiments showed that complement had no effect on inactivation and neutralization of phage by normal and immune sera.

DISCUSSION

Heated normal animal sera contain heat stable factors which inactivate phages of the T-system. Unheated sera have greater activity. The inactivating power of heated and unheated sera increases specifically with immunization. This means that heat stable antibodies increase with immunization. There is a heat stable factor in both normal and immune serum; in the latter it is probably, chiefly, an antibody.

It might be thought that the greater activity of unheated sera was due to an effect of complement on the inactivation or neutralization by the heat stable factor or antibody that is present in normal or immune serum. If this were so, then addition of complement to heated serum should restore its activity to the level found with unheated serum. When normal guinea-pig serum is added as complement to a serum-phage mixture, its own inactivating power must be considered, but the phage-inactivating factor can be removed from normal guinea-pig serum by absorption with the homologous phage, without loss of haemolytic complement. The greater activity of unheated serum was evidently not due to complement, for when this absorbed serum was added as complement to mixtures of phage with heated normal or immune sera, it had no effect. There are evidently both a heat labile inactivating factor in normal serum and a heat labile antibody in immune serum. The fact that these investigations had been carried out on guinea-pigs and rabbits of different laboratories and the same results obtained, led us to the conclusion that the presence of heat labile and heat stable factors in sera of guinea-pigs and rabbits is not connected with the special conditions of the laboratories and experimental animals. Experiments in which fresh additions of phage were made to phage-antiserum mixtures, in which the unneutralized phage had been reduced to 1 % of the initial concentration, showed that the heat labile factor or antibody was exhausted more readily than the heat stable. One addition of phage containing 10^6 phage particles per ml. was sufficient to remove the heat labile activity in equal volume of normal serum without impairing the heat stable activity. The heat labile activity could also be removed from immune serum by phage concentrations which did not affect the heat stable activity. If a quantity of phage more considerable than that required to absorb the heat labile activity is added to the serum, the heat stable activity also is apt to be absorbed. Dilutions of the sera reduced the heat labile activity more than the heat stable activity. When unheated serum is diluted, it loses a much greater proportion of its inactivating power than does the same serum heated. Dilution decreases the concavity of the neutralization curve of unheated serum so that it becomes nearly a straight line. Finally, above a certain dilution both heated and unheated sera had the same effect on the phage. The neutralization curves of highly diluted sera, both unheated and heated, are alike.

SUMMARY

1. Heat labile and heat stable inactivating factors against T-system phages are present in normal guinea-pig and rabbit sera. These may be antibodies.

2. Sera of guinea-pigs immunized with phages T_2, T_3 and T_4 contain heat labile and heat stable antibodies.

3. The heat labile activity is absorbed from guinea-pig serum by phage more readily than the heat stable activity.

4. Dilution of the sera reduces the heat labile activity more than the heat stable activity.

5. Complement has no effect on the inactivation or neutralization of phages T_2, T_3 and T_4 by normal or immune guinea-pig sera.

REFERENCES

ANDREWES, C. H. & ELFORD, W. J. (1933). Observations on anti-phage sera. II. Properties of incompletely neutralized phage. *Brit. J. exp. Path.* **14**, 376.

DELBRÜCK, M. (1946). Bacterial viruses or bacteriophages. *Biol. Rev.* **21**, 30.

GRATIA, A. (1936). Des relations numériques entre bactéries lysogènes et particules de bactériophage. *Ann. Inst. Pasteur*, **57**, 652.

HERSHEY, A. D. & BRONFENBRENNER, J. (1947). Effects of complement on the specific neutralization of bacteriophage. *Fed. Proc.* **6**, 428.

HERSHEY, A. D., KALMANSON, G. & BRONFENBRENNER, J. (1943). Quantitative methods in the study of the phage-antiphage reaction. *J. Immunol.* **46**, 267.

JERNE, N. K. (1956). The presence in normal serum of specific antibody against bacteriophage T_4 and its increase during the earliest stages of immunization. *J. Immunol.* **76**, 208.

JERNE, N. K. & AVEGNO, P. (1956). The development of the phage-inactivating properties of serum during the course of specific immunization of an animal. Reversible and irreversible inactivation. *J. Immunol.* **76**, 200.

KALMANSON, G. M., HERSHEY, A. D. & BRONFENBRENNER, J. (1942). Factors influencing the rate of neutralization of bacteriophage by the antibody. *J. Immunol.* **45**, 1.