# STUDIES ON IMMUNIZATION BY A SPECIES ANTIGEN

## III. THE PRESENCE OF AN ANTI-IMMUNITY FACTOR IN *PNEUMOCOCCUS*

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The first two articles in this series described the presence in pneumococci and in other bacteria of an antigen which on injection excites a general immunity to pneumococci, irrespective of type. This antigen was originally obtained from autolysed pneumococci of different types, and on account of its non-type-specific action was designated 'Species antigen'.

The failure of ordinary bacterial preparations, whether made from pneumococci or from other bacteria, to produce definite cross-immunity to pneumococci is due to the presence of a protein constituent which opposes the immunizing action of species antigen. These two substances, species antigen and the 'opposition factor', are liberated on disintegration of the bacterial bodies and their action is mutually antagonistic. To obtain species immunity readily it is therefore necessary to use a bacterial extract in which the opposition factor is absent or has been rendered comparatively insoluble.

The present investigation deals with the action of the opposition factor in *Pneumococcus*. Is this substance merely antagonistic to species antigen, or is it an anti-immunity factor which reduces resistance and can oppose ordinary type-specific immunization?

## METHODS USED

The type-specific antigen of *Pneumococcus* is contained in the capsule and it dissolves out in the early stages of autolysis. If pneumococci be killed by alcohol and suspended in saline, this antigen can be obtained in a saline solution which is free from other constituents of the organism. The bodies of the pneumococci contain the opposition factor (a protein constituent) and the species antigen, which appears to be of carbohydrate nature. These components are liberated together in the advanced stages of autolysis or by the action of bacterial solvents, but are difficult to separate from each other. If the protein be precipitated, any carbohydrate antigen present is largely or entirely adsorbed to the precipitate. It was found impracticable to obtain the opposition factor free from species antigen.

Hence the experiments to be described were directed to measure any action of the opposition factor against type immunization in excess of that required to neutralize the auxiliary effects of the species antigen. Such a procedure appeared possible because species antigen is present in very small amount compared with the abundance of type-specific capsular material in virulent pneumococci; the ratio may not be more than 1/1000.

Throughout this work, the general methods adopted, the dosages and tests employed were similar to those detailed in the first article of this series; they are summarized in the protocols.

## PRELIMINARY INVESTIGATIONS

The proposed work entailed the measurement of the degree of type immunity excited by the injection of type-specific antigen, and any variation produced by the presence of other pneumococcal constituents. Preliminary investigations were required to ascertain:

(*a*) The dose of intact heat-killed pneumococci which would give an easily measurable degree of type immunity after a single injection.

(*b*) The variations in the immunizing action of pneumococci brought about by a change in the physical state of the type-specific antigen and other constituents—whether in solution or undissolved.

(*c*) Any variation in the immunizing power of type-specific antigen due to the action of chemicals used to secure solution of pneumococci.

### (*a*) *Immunizing value of heat-killed pneumococci*

Type I pneumococci were collected by centrifuge from an 18 hr. serum-broth culture, resuspended in saline of the same volume as the culture, and heated for 1 hr. to 60° C. at *p*H 6·5.

Such a vaccine undiluted contains on an average 500 millions of pneumococci per c.c. Doses of 0·3 c.c. of 1/100, 1/1000 and 1/10,000 dilutions were injected

https://doi.org/10.1017/S002217240003597X Published online by Cambridge University Press

into three sets of mice, which were tested a week later for type immunity against serial dilutions of an 18 hr. culture of virulent organisms.

Table 1

| Type I pneumococci Test doses | Controls | Inoculated with vaccine diluted 1/100 | 1/1000 | 1/10,000 |
|---|---|---|---|---|
| 10 cocci | D | Survived | Survived | D |
| 100 | D | Survived | Survived | D |
| 1000 | D | Survived | Survived | D |
| 10,000 | — | Survived | D | D |
| 100,000 | — | D | D | D |

Hence a 1/1000 dilution of the vaccine, the dose containing about 150,000 heat-killed pneumococci, appeared suitable for detecting variations in response after different treatments. Five similar vaccines were prepared on different occasions and tested; they gave closely similar degrees of protection on injection.

### (*b*) *Immunizing value of pneumococcal type antigen in solution*

For this purpose type I pneumococci collected from an 18 hr. serum-broth culture in two tubes were treated in two ways. One portion was used to make a vaccine as described above. The other portion was resuspended in 50% alcohol, centrifuged down and again suspended in saline, of volume equal to that of the portion of culture used. This suspension was kept 1 day at room temperature and 4 days longer in the refrigerator (4° C.); the *p*H was 6·5. It was then heated to 60° C. for 35 min., cooled and centrifuged apparently clear. Part of this fluid was filtered through a gradocol membrane of 6 $\mu$m. The filtrate gave no precipitate with salicyl-sulphonic acid, but a large precipitate with type I antiserum.

This filtrate was tested for immunizing value, using 0·3 c.c. of dilutions 1/300, 1/1000 and 1/2000 for three sets of mice, which were injected with living organisms 1 week later.

Table 2

| Type I pneumococci Test doses | Controls | Inoculated with filtrate diluted 1/300 | 1/1000 | 1/2000 |
|---|---|---|---|---|
| 10 cocci | D | Survived | Survived | Survived |
| 100 | D | Survived | Survived | Survived |
| 1000 | D | Survived | Survived | Survived |
| 10,000 | — | Survived | Survived | D |
| 100,000 | — | Survived | D | D |

Retested on subsequent occasions the filtrate maintained its original immunizing value. The vaccine made from the same culture gave the same immunity response in 1/1000 dilution as the filtrate in 1/2000 dilution. The unfiltered fluid of the centrifuged autolysate contained enough pneumococci left in suspension to raise its immunizing value to about twice that of the filtrate. Thus 1/2000 dilution of fluid was equal to 1/1000 filtrate.

(*b*2) *Effect of solution or relative insolubility of pneumococcal constituents on their immunizing value.* In accordance with Ehrlich's axiom 'Corpora non agunt nisi soluta', we may expect that the type-immunizing value of pneumococcal preparations will depend largely on the solubility of the type-specific antigen contained. In the following experiment type I pneumococci were resuspended in saline and divided into three portions, each being treated in a different way:

(1) Heated after making well acid (*p*H 4·5) to 60° C. for 1 hr. to harden the cocci.

(2) Autolysed in saline, as in the preceding experiment, heated and centrifuged clear. This preparation contained the type antigen that had very largely dissolved out of the cocci.

(3) Treated with an equal volume of 10% taurocholate. Kept at 37° C. for 30 min., when solution was almost complete. Heated at *p*H 6 to 60° C. for 1 hr. This preparation also contained the type antigen in solution; the other constituents of the pneumococci had been partially dissolved.

These three preparations were diluted with saline to give 1/1000 dilutions of the original portions. They were injected into three sets of mice (dose 0·3 c.c.) which were tested for type immunity 1 week later.

Table 3

| Type I pneumococci Test doses | Controls | Mice inoculated with Cocci acid heated | Fluid of autolysate | Taurocholate preparation |
|---|---|---|---|---|
| 3 cocci | D | — | — | — |
| 9 | D | D | Survived | Survived |
| 90 | D | D | Survived | Survived |
| 900 | — | Survived | D | Survived |
| 9000 | — | D | Survived | Survived |
| 90,000 | — | D | Survived | D |

Hence a solution of type antigen has much greater immunizing value than a comparatively insoluble preparation containing the same amount of antigen.

A converse experiment confirmed this result:

Type I pneumococci were almost dissolved in taurocholate and the preparation then heated at acid *p*H to 60° C. for 30 min. Part of this preparation was treated with 2 vol. of alcohol and the dissolved material precipitated. The precipitate was taken up in saline of equal volume to the part treated.

A 1/2000 dilution of each preparation was injected into two sets of mice (dose 0·3 c.c.) which were tested a week later.

https://doi.org/10.1017/S002217240003597X Published online by Cambridge University Press

Table 4

| Type I pneumococci Test doses | Controls | Mice inoculated with: Taurocholate solution | Mice inoculated with: Alcohol precipitate |
|---|---|---|---|
| 5 cocci | D | Survived | Survived |
| 50 | D | Survived | Survived |
| 500 | D | Survived | D |
| 5000 | — | Survived | D |
| 50,000 | — | Survived | Survived |

Thus precipitation of the type antigen with protein by alcohol produced a mixture from which pure antigen was not absorbed so well.

### (c) *The effect of chemical agents on type-specific antigen*

In a preceding experiment (Table 3) pneumococci heated at *p*H 4·5 suffered a reduction of their immunizing value. Acid heating appeared to render the cocci less soluble, whereas alkali, like taurocholate, has a solvent action. To ascertain whether acid and alkali had any effect on the type-specific antigen itself, the following experiment was performed.

From the filtered saline autolysate of type I pneumococcus (see Table 2) a dilution of 1/100 was made and divided into three parts. One part was treated with an equal volume of *N*/50 NaOH for 1 hr. at room temperature, then neutralized. Another portion was heated with half its volume of *N*/10 HCl (= *N*/30 HCl) for 1 hr. at 60° C., then neutralized with *N*/10 NaOH. From the control third portion and the two treated portions final dilutions equivalent to 1/2000 of the original filtrate were made and injected into three sets of mice (doses 0·3 c.c.). These mice were tested a week later for type immunity.

Table 5

| Type I pneumococci Test doses | Controls | Mice inoculated with filtered autolysate: Untreated | HCl heated | NaOH cold |
|---|---|---|---|---|
| 5 cocci | D | Survived | Survived | Survived |
| 100 | D | Survived | Survived | Survived |
| 1000 | D | D | Survived | Survived |
| 10,000 | — | D | D | D |
| 100,000 | — | D | D | D |

On a subsequent occasion the experiment was repeated and the effect of mild alkali treatment after HCl heating also investigated. Part of the diluted filtrate, after heating in *N*/30 HCl, was over-neutralized and kept at *p*H 9·3 for 2½ hr. Final dilutions 1/2000. (See Table 6).

From these results it appeared that neither hot acid nor short exposure to alkali had a significant effect on the type-specific antigen.

While mild alkali treatment had no deleterious effect on the type-specific antigen, whether applied direct or after acid treatment, longer exposure to *N*/100 NaOH (24 hr. room temp.) or heating in alkali at *p*H 10 or more caused great loss of its immunizing power.

## EFFECT OF OPPOSITION FACTOR

### *The type-immunizing value of an incomplete saline autolysate of* Pneumococcus *is lessened by the presence of undissolved material, but increased by its removal*

After a comparatively short period of autolysis, the bulk of the capsular type-specific antigen passes into solution, leaving the protein undissolved. Should an anti-immunity factor be present in the protein, we might expect injection of the whole autolysate to have less immunizing value than injection of the dissolved antigen. This comparison was made in the following experiment:

Type I pneumococci from an 18 hr. serum-broth culture were collected, treated with 50% alcohol and resuspended in saline of the same volume as the culture. After the suspension had been kept at *p*H 6·5 for 5 days in the refrigerator, it was made more acid (*p*H 5) and heated to 60° C. for 1 hr. Part was then centrifuged clear. The separated fluid, the deposit resuspended in an equal volume of saline, and a portion of the whole autolysate were diluted 1/2000 and each injected into a set of mice (dose 0·3 c.c. each). The mice were tested for type immunity a week later. The experiment was repeated later, using fresh material on other mice, with the results shown in parallel in Table 7.

While injection of the separate fluid portion of the autolysate excited good immunity and the residue some protection, the whole autolysate did not give a superior result but only a partial immunity, paradoxical in nature.

Table 6

| 'ype I pneumococci Test doses | Controls | Mice inoculated with filtered autolysate: Untreated | Cold NaOH | Hot HCl | Hot HCl, warm alkali |
|---|---|---|---|---|---|
| 12 cocci | D | Survived | Survived | Survived | Survived |
| 120 | D | Survived | Survived | Survived | Survived |
| 1200 | — | D | D | D | D |
| 12,000 | — | D | D | D | D |

https://doi.org/10.1017/S002217240003597X Published online by Cambridge University Press

Table 7

| Type I pneumococci Test doses (average) | Controls | Mice inoculated with autolysate: Fluid | | Residue | | Whole | |
|---|---|---|---|---|---|---|---|
| 7 cocci | D | Survived | Survived | Survived | Survived | D | D |
| 14 | D | Survived | Survived | Survived | Survived | D | D |
| 140 | D | Survived | Survived | Survived | Survived | D | D |
| 1400 | — | Survived | Survived | D | D | Survived | Survived |
| 14,000 | — | Survived | D | D | D | D | Survived |
| 140,000 | — | D | Survived | D | D | D | D |

In a subsequent experiment type I pneumococci were autolysed for 2 days at room temperature, and a more complete separation of the type antigen obtained. In 1/2000 dilution the fluid portion excited good type immunity, but the separated residue none. A mixture (whole autolysate) conferred only slight protection.

Table 8

| Type I pneumococci Test doses | Controls | Mice inoculated with autolysate: Fluid | Residue | Whole |
|---|---|---|---|---|
| 11 cocci | D | Survived | D | Survived |
| 110 | D | Survived | D | Survived |
| 1100 | D | Survived | D | D |
| 11,000 | — | Survived | D | D |
| 110,000 | — | D | D | D |

The residual coccal bodies therefore contain some heat-stable substance which reduces the immunizing effect of the type-specific antigen present in the fluid of the autolysate. The action of this opposition factor is strong enough to affect type-specific immunization, besides overcoming the auxiliary effect of the species antigen present in the bodies of the pneumococci.

*The type-immunizing value of heat-killed pneumococci is enhanced by easy solubility of the capsular antigen but impaired by solution of the bacterial bodies*

As shown in a previous experiment (Table 3), pneumococci heated in an acid medium were rendered less soluble and suffered a reduction of their immunizing value, while partial solution by taurocholate made little difference when the preparation was heated.

In the following experiment the immunizing value of pneumococci heated in neutral saline was compared with that of similar cocci heated in dilute acid, and with that of pneumococci finally dissolved in dilute alkali.

Type I pneumococci collected from an 18 hr. serum-broth culture were resuspended in saline of the same volume; from this mother suspension a dilution of 1/50 was made and divided into two parts, (*a*) and (*b*):

(*a*) Diluted with an equal volume of saline and heated to 60° C. for 1 hr. at *p*H 6·8.

(*b*) Treated with half its volume of $N/10$ HCl and heated to 60° C. for 1 hr. Cooled and neutralized with $N/10$ NaOH.

(*c*) A portion of the acid heated suspension after neutralization (Prep. (*b*)) was made well alkaline (*p*H 10·5) and practically dissolved. Neutralized after half an hour.

From these three preparations final dilutions equal to 1/1000 of the original culture volume were made. These were injected into three sets of mice (dose 0·3 c.c.) which were tested for type immunity 1 week later.

Table 9

| Type I pneumococci Test doses | Controls | Mice inoculated with cocci: Neutral heated | HCl acid heated | HCl heated, then alkali |
|---|---|---|---|---|
| 4 cocci | D | Survived | Survived | D |
| 80 | D | Survived | Survived | D |
| 800 | D | Survived | D | D |
| 8000 | — | Survived | D | D |
| 80,000 | — | D | D | D |

The disparity in results is not due to a deleterious effect of the chemicals on the type antigen (Tables 5 and 6), but to changes in the solubility of the pneumococcal constituents. Heating in acid appears to render all the components less soluble, so that type antigen is less readily freed. The subsequent use of alkali has the reverse effect: it dissolves out type antigen and protein. There is sufficient opposition factor thus liberated to neutralize the immunizing action of the antigens.

If pneumococci be heated in $N/30$ HCl and the suspension be then centrifuged, the preparation still contains a not inconsiderable number of suspended cocci, together with some type-specific antigen in solution. Injection of the neutralized preparation in adequate dose (0·3 c.c. of a 1/100 dilution) excites an excellent type immunity. But this response is considerably reduced if the cocci present be largely dissolved by the use of alkali.

The loss of immunizing value of such preparations brought about by the solvent effect of alkali can be fully restored by subsequent reheating at

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acid *p*H. The final acid heating coagulates the protein and renders the opposition factor comparatively insoluble.

Table 10

| Type I pneumococci Test doses | Controls | Acid heated cocci (centrifuged) Neutralized | Alkali treated |
|---|---|---|---|
| 5 cocci | D | Survived | Survived |
| 100 | D | Survived | Survived |
| 1000 | D | Survived | Survived |
| 10,000 | — | Survived | D |
| 100,000 | — | Survived | D |

The results of these experiments confirm those performed with autolysates. Dissolution of the bodies of pneumococci liberates a substance which opposes immunization. This substance can be freed from heated pneumococci and is not a toxic product of autolysis.

*The effect of the anti-immunity factor is limited*

To ascertain the range of interference with immunization by the opposition or anti-immunity factor in pneumococcus, experiments were made with serial dilutions of dissolved vaccine.

A sample of type I *Pneumococcus* heat-killed vaccine, prepared as in former experiments, was almost dissolved by keeping well alkaline (*p*H 11) for 1 hr., then neutralized. From this preparation serial dilutions of 1/10, 1/100 and 1/1000 were made. These and a control sample of untreated vaccine, diluted 1/1000, were injected into four sets of mice (doses 0·3 c.c.) which were tested for type immunity 1 week later. (Table 11.)

Table 11

| Type I pneumococci Test doses | Controls | Mice inoculated with heated pneumococci: Untreated 1/1000 | Dissolved with alkali 1/1000 | 1/100 | 1/10 |
|---|---|---|---|---|---|
| 16 cocci | D | Survived | D | Survived | Survived |
| 160 | D | Survived | D | D | Survived |
| 1600 | — | Survived | D | Survived | Survived |
| 16,000 | — | D | D | Survived | Survived |

It is thus evident that a sufficiently large dose of type-specific antigen is able to produce immunity in spite of an equally large dose of opposition factor. The unbalanced effect suggests that the antigen and the opposition substance do not act altogether on the same mechanism, but may affect different factors concerned in the production of immunity.

Since alkali-dissolved vaccine in a small dose (0·3 c.c. of 1/1000 dilution) excited no immunity, the effect of two doses given at a week's interval was tested. The repetition failed to evoke an immunity response; it is doubtful whether any antibody to the opposition factor is formed.

The injection of a preparation which contained opposition factor but little type antigen was found to reduce the resistance of mice, as compared with that of ordinary controls, when tested with pneumococci of reduced virulence.

*Destruction or inactivation of the anti-immunity factor*

In the experiments on pneumococcal autolysates in saline, the fluid portion was found to contain most of the type-specific antigen. The residual bodies contained a little undissolved type antigen together with the species antigen and the protein containing the opposition or anti-immunity factor.

In Tables 7 and 8, comparison of the fluid portion with the whole autolysate showed that the undissolved portion reduced the immunizing response excited by the dissolved antigen in the fluid.

It seemed probable that a more striking result could be obtained if the residue portion were deprived of its antigen content. A more concentrated preparation of the opposition factor would then be available for the study of its effect on type immunization.

For this purpose the residual cocci collected from a pneumococcal autolysate were dissolved in weak NaOH and then heated to 60° at *p*H 9·7 for 50 min. to destroy or inactivate the antigens. But although this solution conferred no immunity when tested alone, neither did it reduce the immunizing response when injected together with the fluid portion of the autolysate, 1/2000 dilution. The experiment was repeated, using a weaker dilution of the antigenic autolysate fluid (1/10,000) and a stronger solution of the alkaline heated bodies (1/10).

Table 12

| Type I pneumococci Test doses | Controls | Mice inoculated with pneumococci autolysate: Fluid only 1/10,000 | Fluid plus residue 1/10 | Residue only 1/10 |
|---|---|---|---|---|
| 10 cocci | D | Survived | Survived | D |
| 200 | D | D | Survived | D |
| 2000 | D | Survived | Survived | D |
| 20,000 | — | D | Survived | D |
| 200,000 | — | D | D | D |

https://doi.org/10.1017/S002217240003597X Published online by Cambridge University Press

While the solution of the residual cocci excited no immunity response by itself, it actually enhanced the effect of the very small dose of type antigen when injected in combination. This effect is unusual; repetitions of the experiment generally gave equal measures of protection from the fluid alone and from the mixture. The disappearance of any anti-immunity action showed that the opposition factor as well as the antigens had been destroyed or inactivated by alkaline heat. Provided the solution be kept acid, heating to 100° C. has no destructive effect.

## SUMMARY

The bodies of pneumococci contain a constituent of protein origin which interferes with immunization.

This constituent can not only counteract the immunizing action of the species antigen, but reduce the immunity response to the type-specific antigen.

In heat-killed pneumococci (ordinary bacterial vaccine) the coagulation of the protein renders the anti-immunity factor relatively insoluble and minimizes its action.

In solutions of pneumococci the anti-immunity factor is liberated and reduces the immunity response to the type-specific antigen.

The immunizing effect of a small dose of heat-killed pneumococci can be abolished if the cocci be dissolved before injection. But a larger dose of dissolved vaccine produces immunity, the effect of the antigen predominating.

The anti-immunity factor is heat resistant at acid *p*H, but is destroyed by alkaline heat. In these respects it resembles the immunizing antigens.

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(*MS. received for publication* 7. v. 44.—Ed.)