THE EFFECT OF A TISSUE ENZYME UPON PNEUMOCOCCI

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Rabbits immunized by the intravenous injection of heat-killed encapsulated pneumococci respond by the production of the type specific antibodies directed against the capsular polysaccharide of the cell used as antigen. When, however, the same antigen is injected into animals of the same species by the intradermal route (skin of the mid-flank) the type specific antibodies fail to appear (1–3). It appeared possible that when pneumococci are injected into the skin of the rabbit, they are subjected to the action of certain tissue enzymes which inactivate the type specific antigen. It is known in fact that this antigen is readily inactivated by the autolytic ferments of the bacterial cell (4, 5); these ferments however, are completely destroyed by heating in the course of preparation of the bacterial antigen. The present paper reports the existence of an enzyme, widely distributed in animal tissues, which modifies the staining properties and the antigenicity of heat-killed pneumococci.

EXPERIMENTAL

Cultures.—Virulent pneumococci were grown and preserved in blood broth and passed through mice often enough to maintain a degree of virulence such that 0.000,000,01 cc. of an 8 hour culture would kill 20 gm. mice within 72 hours. Cultures of R variants were similarly grown in blood broth.

Heat-Killed Cells.—Pneumococci recovered from a young broth culture were resuspended in a small amount of distilled water and rapidly added to a larger volume of distilled water at 75°C.; the temperature was maintained at this level for 20 minutes. This process of "flash" heating was selected because it minimizes the chances of alterations due to autolytic action (6).

Method of Immunization.—The cell suspensions were injected into rabbits intravenously on 6 consecutive days followed by a free interval of 1 week. Three courses were given, each animal receiving daily a dose containing the cells from 1 cc. of broth culture. The rabbits were bled on the 8th day after the last injection of each course.

Serological Tests.—In all precipitin reactions, 0.5 cc. of immune serum or 0.2 cc. of serum diluted to 0.5 cc. with salt solution, was added to an equal volume of graded dilutions of the purified capsular polysaccharide. The mixtures were incubated for 2 hours at 37°C.; final readings were made after keeping the reacting mixtures overnight in the ice box.

Preparation of the Enzyme.—Methods of extraction of the enzyme from polymorphonuclear leucocytes are described in the present paper. The purification of the enzyme extracted from dried pancreatin, and determination of its chemical activity, have been described elsewhere (7).

The Fate of Heat-Killed Pneumococci Injected into the Skin of the Rabbit.—As stated above, heat-killed encapsulated pneumococci injected into the skin of the rabbit fail to stimulate the formation of the type specific carbohydrate antibodies which arise in response to the intravenous injection of the same antigen. An attempt was therefore made to follow by microscopic examination the fate of pneumococci injected into the skin, in the hope of finding a clue to the mechanism of lack of antigenic response.

Experiment 1.—0.1 cc. of a suspension of heat-killed pneumococci (Type I) was injected at several sites into the skin of a rabbit. The injected areas were excised at different intervals of time, and films, made from the tissue fragments, were stained by the Gram technique.

There was, of course, a pronounced polymorphonuclear infiltration at the sites of injection, but at first only little, if any, phagocytosis. The pneumococci, however, were seen to undergo a process of extracellular digestion which began within 24 hours after injection, and was completed in 4 to 5 days; many bacteria became Gram-negative before being engulfed by the leucocytes. These morphological observations suggested that the pneumococci were being attacked by ferments released at the site of injection.

The Effect of Leucocytic and Other Tissue Enzymes on the Morphology of Heat-Killed Pneumococci.—Normal rabbit skin was ground up, then extracted under a wide range of conditions in an attempt to separate the principle responsible for the change in staining reaction of the injected pneumococci (Experiment 1).

The extracts of normal skin exhibited marked enzymatic activity when tested with a number of soluble substrates (proteins, phosphoric esters, fats, etc.) but completely failed to exert any appreciable action on the bacterial cells.

The presence of an inflammatory reaction at the site of inoculation suggested that leucocytes released soluble ferments capable of attacking the pneumococci. A number of experiments were instituted to determine the validity of this assumption (Experiment 2).

Experiment 2.—A sterile, polymorphonuclear exudate was obtained by injecting aleuronate into the pleural cavity of a normal rabbit. The exudate, consisting almost exclusively of polymorphonuclear cells, was collected after 24 hours. The cells were washed, resuspended in saline, allowed to autolyze at 37°C. for 48 hours, then extracted with N/10 acetic acid for 24 hours. The extract was heated at boiling temperature for 10 minutes and the coagulum removed by centrifugalization; the new precipitate which formed on neutralization of the supernate was also discarded. The material was then dialyzed, and again centrifuged; the clear soluble fractions were used as enzyme. 0.1 cc. of purified leucocytic extract was added to 1 cc. of a suspension of heat-killed pneumococci at pH 7.0; the mixture was incubated at 37°C. for 24 hours. Films made from this preparation were stained with the eosin-methylene blue stain or by the Gram technique.

Whereas the control untreated pneumococci were intensely stained by methylene blue, and reacted positively to the Gram stain, the cells treated with the leucocytic extract had lost all affinity for the basic dyes and appeared as faint red structures when counterstained with eosin or safranin. The cell bodies, however, were not disintegrated but on the contrary retained their characteristic morphology. It is also worth noting that there was no other evidence of lysis, since the bacterial suspension lost only little of its original turbidity.

The same changes in staining characters can be observed when R or S variants of pneumococci, irrespective of type derivation, are treated with the leucocytic extract. The results are also identical when the bacterial cells are killed by methods other than heating, such as treatment with formaldehyde and acetic acid.

Similar extracts, capable of destroying the affinity of pneumococci for the basic dyes, but without causing any disintegration of the cell structure, have been obtained from the organs—in particular the liver, pancreas, spleen and lungs—of several animal species. The same enzyme has also been prepared from the pleural exudate of a tuberculous patient, who developed empyema following injection with *Hemophilus influenzae*.

All the enzyme preparations have a number of properties in common, irrespective of the source from which they are obtained. The

active principle is heat resistant, especially at slightly acid reactions; its rate of activity upon pneumococci increases with temperature up to 70°C.; its range of activity is between pH 5.0 and pH 9.5; it is completely resistant to trypsin and chymotrypsin, but is rapidly inactivated by pepsin; it gives the common protein reactions. Finally all the preparations exhibit a high degree of enzymatic activity on yeast nucleic acid.

It may be noted that all these properties are the same as those of the purified nuclease (prepared from dried pancreatin) which has been described elsewhere (7). In fact, the purified nuclease from this source is also capable of attacking pneumococci, giving rise to the same alterations in staining reactions brought about by the leucocytic enzyme described above.

Effect of the Leucocytic Enzyme on the Antigenicity of Encapsulated Pneumococci.—Heat-killed encapsulated pneumococci, injected into rabbits by the intravenous route, elicit the production of the specific antibodies directed against the capsular polysaccharide of the cell used as antigen. The effect of the leucocytic enzyme on this "capsular antigen" is determined in the following experiment.

Experiment 3.—A purified extract obtained from the pleural exudate of a tuberculous patient who had developed empyema following secondary infection with *H. influenzae* was prepared according to the method described in Experiment 1. 10 cc. of this extract (at pH 7.0) were added to an equal volume of suspension of heat-killed Type I pneumococci. The mixture was incubated at 37°C. for 38 hours. Some of the bacterial suspension diluted with saline at pH 7.0 without the addition of active enzyme was also incubated to serve as control. Both suspensions were centrifugalized at the end of the incubation period, and the cells resuspended in saline were used for the immunization of two groups of 3 rabbits each by means of the intravenous route. The animals were bled after the third course of immunization and their sera were tested for the presence of type specific precipitins and agglutinins.

Stained films of the two bacterial suspensions used for immunization showed the control cells to be strongly basophilic and Gram-positive whereas over 95 per cent of the cells treated with enzyme had lost their affinity for the basic dyes; the treated cells, however, did not show any evidence of cellular disintegration or lysis.

The 3 rabbits immunized with the Gram-positive cocci developed in their sera precipitins for the Type I polysaccharide; type specific

agglutinins also appeared in respective titers of 1:320, 1:80, 1:80. Of the 3 rabbits immunized with the enzyme-treated cells, one showed in its serum weak precipitins for the Type I polysaccharide; the other 2 were negative. The type specific agglutinins were also much reduced, the titers being respectively 1:20, 1:2, 1:2.

Five similar experiments were performed. In all cases there was marked reduction, or complete absence, of the type specific precipitins and agglutinins in the sera of animals immunized with the enzyme-treated cells, as compared with the sera of animals immunized with control encapsulated pneumococci. It was, however, found impossible to obtain bacterial suspension in which, after treatment with the enzyme, all the cells had lost their basophilic property. A small percentage of intact cells persisted in all cases and these cells probably accounted for the slight residual type specific antigenicity of the bacterial suspensions treated with the leucocytic enzyme, since it is known that heat-killed cells of Type I pneumococci constitute an antigen active in minute amounts.

Nature of the Action of the Leucocytic Enzyme on Pneumococci.— It has been shown that the action of the leucocytic enzyme on pneumococci can be recognized by two types of alteration: (*a*) destruction of the basophilic properties of the bacterial cell; (*b*) inactivation of the capsular polysaccharide antigen. A complete understanding of these findings depends of course on a knowledge of the chemical nature of the substrates attacked by the enzyme. The enzyme, although it inactivates the capsular polysaccharide antigen does not attack the capsular polysaccharide itself. In fact, of all of the soluble substrates tested, yeast nucleic acid is the only one to be attacked by the purified preparations. An analysis of this reaction is presented elsewhere (5).

The Gram-positive structure of heat-killed pneumococci is completely resistant to crystalline pepsin, but it is slowly attacked by some preparations of crystalline trypsin and chymotrypsin. It was found, however, that the same preparations of crystalline trypsin or chymotrypsin which slowly reduce the basophilic properties of pneumococci also exhibit some enzymatic activity against yeast nucleic acid. With repeated recrystallizations of the proteolytic enzymes, however, the activity against pneumococci and nucleic acid eventually

disappears, although the proteolytic activity remains unimpaired.[1] It is evident therefore that the loss of the basophilic character of the cell is not associated with proteolysis. These observations also emphasize once more the close correlation which exists between the ability of the enzyme preparations to decompose yeast nucleic acid and to attack the bacterial cell.

DISCUSSION

It is possible to extract from washed polymorphonuclear leucocytes, and from many animal tissues, an enzyme capable of destroying the basophilic character of heat-killed pneumococci, and of inactivating the capsular polysaccharide antigen of encapsulated cells of the same species. The enzyme, however, does not decompose the capsular polysaccharide itself, and since it attacks both R and S variants, the point of attack of the bacterial cell by the enzyme must be a structure common to all pneumococci. Of all soluble substrates tested, yeast nucleic acid is the only one to be decomposed by the purified enzyme preparations; it is also true that a purified nuclease obtained from dried pancreatin attacks both the basophilic structure and the capsular antigen of pneumococci. It is therefore possible that the cellular structure attacked by the enzyme is related to nucleic acid; this point of view will be substantiated by a description to be presented in a later publication of the products released in solution when the enzyme attacks the bacterial cell. No explanation is available at the present time of the mechanism whereby the encapsulated cell which loses its basophilic character, loses at the same time its effectiveness as type specific antigen. It is worth repeating in this connection that the enzyme does not cause any lysis or disintegration of the cell body, nor does it decompose the capsular polysaccharide.

The question which prompted the present study was to account for the failure of rabbits to develop type specific antibodies following the intradermal injection of heat-killed encapsulated pneumococci. It was observed that dead pneumococci injected into the skin remain at the site of injection for several days and bring about an accumulation of polymorphonuclear leucocytes; it was also demonstrated that these

[1] We are greatly indebted to Dr. J. H. Northrop and Dr. M. Kunitz for supplying samples of crystalline trypsin and chymotrypsin recrystallized several times.

leucocytic cells contain an enzyme which inactivates the capsular antigen *in vitro*. The change in staining reaction of the injected pneumococci suggests that the same enzymatic action may take place *in vivo* and account, partly at least, for the lack of type specific antigenic response to the intradermal injection of the bacterial cells.

SUMMARY

Polymorphonuclear leucocytes contain an enzyme which destroys the basophilic character of heat-killed pneumococci (R and S variants) and inactivates the type specific polysaccharide antigen of encapsulated cells. The same enzyme, however, fails to cause a disintegration of the bacterial cells, or to decompose the capsular polysaccharide itself.

The enzyme has been extracted from a number of animal tissues; it appears identical with a purified enzyme extracted from pancreatin and which decomposes yeast nucleic acid.

These facts are considered with regard to the failure of rabbits to produce the type specific carbohydrate antibodies when immunized with heat-killed encapsulated pneumococci by the intradermal route.

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