# ASSOCIATION OF "NATURAL" ANTIBODIES TO GRAM-NEGATIVE BACTERIA WITH THE $\gamma_1$-MACROGLOBULINS*

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Natural or normal antibodies are defined as the antibodies which are universally encountered in the sera of all mammalian species in the absence of apparent infection or immunization. The nature of these antibodies, their origin, specificity, and chemical characteristics have been the subject of much controversy in the past. The distinction between natural and immune antibodies was principally based on apparent differences in specificity and heat stability. It was shown recently that there are no differences in specificity between natural and immune antibodies; however, certain physicochemical differences between the two types of antibodies have been observed (1).

It is now widely accepted that the immune globulins can be divided by their electrophoretic, ultracentrifugal, and antigenic properties into 4 groups, the 7S $\gamma_2$-globulin, 7S $\gamma_1$-globulin, 19S $\gamma_1$-globulin, and $\sim$1S globulin (2). It has recently been demonstrated that most of the bactericidal antibodies to Gram-negative organisms are not transferred from maternal sera to the newborn human infant by virtue of the fact that these antibodies are principally found in the 19S $\gamma_1$-globulin fraction (3). It was also reported that sera from several patients with dysgammaglobulinemia, characterized by a very high serum concentration of 19S $\gamma_1$-globulin and virtually no 7S $\gamma$-globulin, contained extraordinarily high titers of bactericidal antibodies; these antibodies are totally absent from the sera of patients with congenital agammaglobulinemia (4).

These findings suggested that the antibodies to Gram-negative bacteria encountered in the random sampling of normal human population are present principally in the 19S $\gamma_1$-globulin fraction of serum and that they may differ from immune antibodies by virtue of their belonging to different molecular species. The $\gamma_1$-macroglobulin was isolated from Cohn fraction III-I of pooled human plasma and the amount of bactericidal, hemagglutinating, mouse-

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protective, and opsonophagocytic antibody activities in this fraction were estimated and compared quantitatively with the antibody activities found in Cohn fraction II of pooled human plasma.

## *Materials and Methods*

*Cultures.*—*Salmonella typhosa* 0901 and *Escherichia coli* 0127:B8 were originally obtained from the culture collection of the Walter Reed Army Institute for Research, Washington, D. C., and subsequently maintained in this laboratory for over 2 years. A culture of *Pseudomonas aeruginosa* isolated from a child with cystic fibrosis and a culture of *Klebsiella pneumoniae* isolated from a child with chronic bronchiectasis were obtained from the diagnostic laboratory of the Children's Hospital.

*Bactericidal Tests.*—The bactericidal tests were performed by a method previously described (1). In summary, 0.1 ml of the test bacterial suspension ($\sim 2 \times 10^8$), 0.3 ml of fresh human serum diluted 1:5 and absorbed at 0°C with a heavy suspension of the test organism, and 0.1 ml of serial dilutions of antibody were incubated for 1 hour at 37°C after which time 2 ml of 2 per cent nutrient agar was added to the incubation mixture. Following incubation overnight at 37°C the number of bacterial colonies in the agar were counted.

*Hemagglutination Tests.*—Hemagglutination tests were carried out as previously described (5). Human red cells, group O, were washed three times in saline and then adjusted to a concentration of 10 per cent. The cells were sensitized by incubation for 1 hour at 37°C with boiled endotoxin or a saline extract of boiled bacteria. The sensitized cells were washed three times in saline and diluted to a 2 per cent suspension. 0.2 ml of the sensitized cells were incubated for 2 hours at 37°C with an equal volume of antibody dilution. The hemagglutination titer was determined by visual inspection after storing the tubes overnight at 4°C.

*Mouse Protection Tests.*—A modification of Shilo's method was used (6). White female Swiss mice, each weighing 20 gm, were obtained from the Charles River Farms, Brookline Massachusetts. Each mouse was injected intravenously first with 0.1 ml saline solution containing 5 mg of levan (kindly supplied by Dr. W. Firshein, Hebrew University, Jerusalem, Israel) and then with an intraperitoneal injection of 0.1 ml suspension of $\sim 1 \times 10^7$ bacteria from a 3-hour-old culture of *S. typhosa 0901*. Immediately thereafter 0.1 ml of globulin diluted in saline was injected intraperitoneally. The number of mice surviving were counted after 48 hours.

*Opsonophagocytic Tests.*—An inoculum of *S. typhosa 0901* was grown overnight at 37°C in 250 cc of brain-heart nutrient medium to which 50 microcuries of $I^{131}$-labeled 5-iodo-2'-deoxyuridine (kindly supplied by Dr. W. L. Hughes, Brookhaven National Laboratory, Upton, New York) had been added. The bacterial harvest was washed three times in 250 ml of sterile saline and repeatedly centrifuged at 8000 RPM for 20 minutes. The supernatant fluid from the final washing was found to be without detectable radioactivity. The labeled bacteria were diluted prior to injection in sterile saline so that each mouse received approximately $1 \times 10^7$ of the bacterial suspension in a 0.2 ml volume. Groups of 3 mice were bled at varying intervals by severing the brachial artery and 1 ml of the pooled blood of each group of mice was assayed for radioactivity in a well scintillation counter. Less than 1 per cent of the injected label was not precipitable with an equal volume of 20 per cent TCA. The assays of the blood samples were corrected for background and the results were expressed as corrected counts/minute/ml of blood. The $K$ value for the slope of the bacterial clearance rate was determined by the method of Benacerraf (7) so that $K = \log C_1 - \log C_2/T$.

*Antibodies.*—Pooled human $\gamma_2$-globulin (Cohn fraction II) was obtained from E. R. Squibb and Sons, New York, (lot 352-1) as a 16.5 per cent solution.

The $\gamma_1$-macroglobulin was prepared from Cohn fraction III-I of pooled human plasma (kindly supplied by Dr. R. Pennell, Protein Foundation, Jamaica Plain, Massachusetts).

Freshly prepared fraction III-I was dissolved in bicarbonate buffer at pH 7.3. The solution was dialyzed against 8 per cent sucrose in phosphate-buffered saline at pH 7.6 in the cold for 18 to 24 hours. The dialyzed fraction was then layered on 10 to 40 per cent sucrose gradients (8) and centrifuged at 35,000 *rpm* in a SW 39L swinging bucket head in a Spinco model L ultracentrifuge for 14 hours at 4°C. The tubes were sliced so that the bottom 1 cm (of 5 cm) of the gradient was collected and dialyzed in the cold against saline buffered to pH 7.6 with sodium phosphate. The macroglobulin prepared in this manner gave a single ultracentrifugal

TABLE I

*Bactericidal Activity of $\gamma_1$-Macroglobulin and $\gamma_2$-Globulin*

| Type of preparation | Amount of preparation required for 50 per cent killing of the test strain inoculum | |
|---|---|---|
| | *S. typhosa* 0901 | *E. coli* 0127 |
| | *mg* | *mg* |
| $\gamma_1$-Macroglobulin | $7.2 \times 10^{-3}$ | $1.8 \times 10^{-3}$ |
| $\gamma_1$-Macroglobulin absorbed with *S. typhosa* | 1 | $1.8 \times 10^{-3}$ |
| $\gamma_1$-Macroglobulin absorbed with *E. coli* | $7.2 \times 10^{-3}$ | 1 |
| $\gamma_2$-Globulin | 1 | 1 |

TABLE II

*Hemagglutinating Activity of $\gamma_1$-Macroglobulin and $\gamma_2$-Globulin*

| Bacterial species | Amount of preparation required for minimal degree of macroscopic hemagglutination | |
|---|---|---|
| | $\gamma_1$-Macroglobulin | $\gamma_2$-Globulin |
| | *mg* | *mg* |
| *Shigella flexneri* | $0.9 \times 10^{-3}$ | $2.5 \times 10^{-1}$ |
| *Klebsiella pneumoniae* | $0.9 \times 10^{-3}$ | $2.5 \times 10^{-1}$ |
| *Pseudomonas aeruginosa* | $1.8 \times 10^{-3}$ | $2.5 \times 10^{-1}$ |
| *Escherichia coli* 0127 | $1.8 \times 10^{-3}$ | $2.5 \times 10^{-1}$ |
| *Salmonella typhosa* 0901 | $4.5 \times 10^{-3}$ | $2.5 \times 10^{-1}$ |

peak with a sedimentation constant of 19S and a single band of precipitation upon immunoelectrophoresis in the $\beta_2$M region. Gamma globulin concentrations in the fraction II and macroglobulins of fraction III-1 were estimated spectrophotometrically and by a previously described immunochemical method (9).

## RESULTS

*Bactericidal Activity.*—Serial dilutions of $\gamma_1$-macroglobulin and $\gamma_2$-globulin preparations obtained from pooled human plasma were tested in the bactericidal system and the results of the experiments are shown in Table I. It was found that in the presence of 0.0072 mg and 0.0018 mg of the macroglobulin preparation 50 per cent of the *S. typhosa* 0901 and *E. coli* 0127 inocula respec-

tively were killed, whereas 1 mg of the $\gamma_2$-globulin preparation was required to achieve the same effect. The specificity of antibodies for *E. coli* and *S. typhosa* was verified by absorbing the macroglobulin preparation with the test organisms for 1 hour at 4°C. The absorbed macroglobulin preparation lost antibody

TABLE III

*Protective Effect of $\gamma_1$-Macroglobulin and $\gamma_2$-Globulin in Mice Challenged with Salmonella typhosa 0901*

| Preparation | Amount of globulin injected per mouse | No. of survivors tested 48 hours after challenge with $10^7$ bacteria |
|---|---|---|
| | *mg* | |
| $\gamma_1$-Macroglobulin | 2 | 5/5 |
| | 1 | 5/5 |
| | 0.5 | 5/5 |
| | 0.2 | 2/5 |
| | 0.15 | 0/5 |
| $\gamma_2$-Globulin | 10 | 5/5 |
| | 5 | 2/5 |
| | 2 | 1/5 |
| | 1 | 0/5 |

TABLE IV

*Opsonophagocytic Effect of $\gamma_1$-Macroglobulin and $\gamma_2$-Globulin on Clearance of $I^{131}$-Labeled S. typhosa 0901 in Mice*

| Preparation | Amount of globulin mixed with bacteria | *K* value | Dose cleared in 3 minutes |
|---|---|---|---|
| | *mg* | | *per cent* |
| Saline control | 0 | 0.041 | 40 |
| $\gamma_1$-Macroglobulin | 0.3 | 0.067 | 85 |
| | 0.15 | 0.043 | — |
| $\gamma_2$-Globulin | 1.0 | 0.066 | 55 |
| | 0.5 | 0.046 | — |
| | 0.25 | 0.042 | — |

An inoculum of $1 \times 10^7$ of labeled *S. typhosa* was injected intravenously per mouse.

activity to the homologous organism used for absorption and retained undiminished antibody activity to the heterologous bacterium.

*Hemagglutinating Activity.*—The hemagglutinating antibodies of the $\gamma_1$-macroglobulin and $\gamma_2$-globulin fractions were tested utilizing human group O red cells coated with the somatic antigens of five representative strains of Entero-

bacteriaceae. In these experimente the minimal amounts of the $\gamma_1$- and $\gamma_2$-globulin preparations required to cause macroscopic hemagglutination were determined; the results are shown in Table II. The magnitude of the differences between the amounts of hemagglutinating antibodies present in the $\gamma_1$-macroglobulin and $\gamma_2$-globulin fractions is in order of 50- to 100-fold. The amounts of $\gamma_1$-macroglobulin needed to cause hemagglutination of any one of the 5 Enterobactericeae tested were similar, indicating that the levels of "natural" antibodies specific for Gram-negative species found in human sera are of the same order.

*Mouse Protection Tests.*—White female mice were pretreated with 5 mg (0.1 ml) of levan by intravenous injection. The mice were given $1 \times 10^7$ *S. typhosa* 9091 intraperitoneally 15 minutes later; the dose selected was found to be uniformly lethal for the strain of mice used. Immediately after the injection of the bacteria, 0.1 ml of various dilutions of the $\gamma_2$-globulin of $\gamma_1$-macroglobulin was also injected into the peritoneal cavity. The number of animals surviving 48 hours after the bacterial challenge was counted; the results are shown in Table III. It was found that 10 mg of $\gamma_2$-globulin were required for complete protection of the mice, whereas the injection of 0.5 mg of $\gamma_1$-macroglobulin resulted in total survival of the group.

*Opsonophagocytic Tests.*—$I^{131}$-labeled *S. typhosa* 0901 were opsonized with various dilutions of $\gamma_2$-globulin or $\gamma_1$-macroglobulin and then injected intravenously into mice. Control mice were injected with labeled bacteria diluted in saline. Radioactivity of blood samples collected at 5 and 15 minutes after injection was assayed for determination of $K$ values as described in the section on Materials and Methods. The $K$ values obtained with labeled *S. typhosa* 0901 after opsonization with immune globulins are given in Table IV. The differences in the effect of the two classes of globulins in opsonophagocytic tests, as expressed by the $K$ values, are not as large as those found in the other test systems. This discrepancy may be due to the fact that determination of the $K$ values was based on the bleedings at 5 and 15 minutes because this interval represented the straight part of the curve. In our experiments, at 5 minutes the major part of the bacteria were cleared from the blood stream. After 3 minutes 40 per cent of the bacteria were cleared in the control animals. When mixed with 1 mg of $\gamma_2$-globulin, 55 per cent of the labeled bacteria were cleared in 3 minutes, and 85 per cent of bacteria opsonized with 0.3 mg of $\gamma_1$-macroglobulin were cleared during the same interval.

## DISCUSSION

This study has revealed that the antibodies to the somatic antigen of the Gram-negative bacteria found in pooled human plasma are principally associated with the $\gamma_1$-macroglobulin of Cohn fraction III-I. As measured by bactericidal, hemagglutinating, mouse-protective and opsonophagocytic tests,

the $\gamma_1$-macroglobulin of human plasma contains up to 100 times more antibody on a weight basis against Enterobacteriaceae than that found in $\gamma_2$-globulin of pooled human serum.

Enders first showed the presence of agglutinins to the O antigen of *S. typhosa* in Cohn fraction III-I of human serum (10). Shilo found mouse-protective substance in bovine fraction III-I but he could not identify the nature of the protective substance (6). Jenkin and Rowley more recently associated opsonophagocytic substances against *Salmonella typhimurium* with a high molecular weight globulin in pig serum (11). On the basis of their inability to detect a decrease in the amount of this high molecular weight globulin by specific absorption they were unable to conclude whether the opsonophagocytic substance was a specific antibody. As the $\gamma_1$-macroglobulin fraction is composed of a very heterogeneous antibody population, it is not surprising that an undetectable quantitative change occurs upon absorption with a single bacterial organism.

The predominance of the antibacterial antibodies in the $\gamma_1$-macroglobulin fraction of normal human serum probably results from the low level of antigenic stimulation provided by the normal environment, in the absence of overt infection (1). We have observed that the serum of germ-free mice contained only 19S bactericidal antibody prior to any immunization. Following repeated injections of *S. typhosa* both 7S and 19S bactericidal antibodies were present in the sera of these animals (12). Svehag and Mandel have shown that repeated stimulation with small amounts of poliomyelitis virus results in the synthesis of only 19S $\gamma_1$-globulin antibody with viral neutralizing activity in rabbits. They found that the production of 7S $\gamma_2$-globulin antibody was evoked only in response to larger doses of antigen (13). Their findings have been confirmed by Uhr and Finkelstein with $\phi$X 174 phage (14).

After fractionating the sera from 40 pregnant women, we found that only 10 per cent of these sera contained any 7S antibody against *E. coli* or *S. typhosa* whereas all 40 sera contained 19S bactericidal antibody against these organisms (3). This distribution of antibody in a normal population accounts for the relatively small quantity of antibody against the Enterobacteriaceae in Cohn fraction II from pooled human plasma. The findings of Neter *et al.* (15) and Fisher and Manning (16) that $\gamma_2$-globulin of fraction II contains antibodies against the Gram-negative bacteria were confirmed, but it is obvious that large amounts of pooled 7S $\gamma_2$-globulin are required to obtain such activity by any of the methods used while much smaller amounts of pooled $\gamma_1$-macroglobulin contain similar antibody activity.

Of the four methods used in this study, the bactericidal and hemagglutination tests were the most sensitive for the detection of antibody. The magnitude of differences in amount of antibody contained in the $\gamma_2$-globulin and $\gamma_1$-macroglobulin was approximately the same for the bactericidal, hemagglutination, and mouse-protective tests. The results obtained in the opsonophagocytic tests were not of the same magnitude when the clearance in the blood of the mice was assayed at 5 and 15 minutes after injection of labeled bacteria. By selecting earlier time periods for the bacterial clearance, a more pronounced difference in the opsonizing effects of the 7S $\gamma_2$-globulin and the 19S $\gamma_1$-globulin was demonstrated, consistent with the differences found utilizing the other three methods of measurement. The kinetics of bacterial clearance cannot in

fact be described by a straight line derived from the determination of the $K$ value between two arbitrarily chosen intervals as indicated by Biozzi *et al.* (16). It appears from the consistency in differences observed between the antibody content of the 7S $\gamma_2$- and 19S $\gamma_1$-globulin that the activity of the same antibodies to one or more cell wall antigens was measured by all the methods employed.

In light of the findings presented it is suggested that the terms "natural" and "normal" antibody are irrelevant and that more precise terminology based on the physicochemical properties of the antibodies under discussion should be substituted in the future.

SUMMARY

"Natural" antibodies to representative species of Enterobacteriaceae were found to be principally associated with the $\gamma_1$-macroglobulin prepared from Cohn fraction III-I of pooled human plasma. The amount of antibodies present was estimated by measuring the bactericidal, hemagglutinating, mouse-protective, and opsonophagocytic activities of this fraction. Comparison with the antibody activity of $\gamma_2$-globulin of Cohn fraction II showed that up to 100 times more antibody activity on a weight basis was present in the macroglobulin fraction. These findings suggest that differences between natural and immune antibody result from the physicochemical properties of 2 different classes of immune globulin.

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