The Physiological Role of the Lymphoid System* **

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There are two recognized functions of the lymphoid system that have been elegantly exploited in the research activities of numerous laboratories during the past few decades. The circulatory function and its attendant transport of cells and metabolite, has been the subject of unabated anatomical and physiological study. The immunological function has witnessed a series of explosive advances rivalled only by the renaissance of molecular genetics.

The immunological role is clearly related to the defense mechanism of the body through various reactions involving the antibody molecule. It has always been a source of mystery to me how nature would construct a series of elaborate organs, the lymphoid system, with the anticipatory function of arresting or fending off any possible invasion of the body by destructive organisms or their toxic products. There is no obvious parallel known in the evolutionary process.

It is more in keeping with biological principles to assume that this important immunological function appeared sometime during the evolutionary process as a useful outgrowth of a more fundamental and necessary physiological function. There are numerous and sufficient examples that one can cite in support of this. The detoxification functions of the liver, acetylation, methylation, glucuronidation and sulfation, while perhaps helpful for survival, nevertheless are recognized as important steps in the physiology and metabolism of the animal. Less sophisticated examples can be singled out among many that bear on this point. The effective horn of the rhinoceros that has similar defensive value against larger enemies is a modified outgrowth of the epidermal organ. The deciduous antlers of the cervine and the permanent horns of the bovine are similar examples of skeletal participation in the defense against predators.

During the past few years a series of studies in our laboratory, dealing with the mechanism of antibody-antigen interaction, led us to propose a new concept (1) which culminated in the demonstration of an important physiological role of the lymphoid system. The primary function of this system of organs is the elaboration of specific γ-globulins that bind to the complementary receptor sites on the cell membrane and affect its structural integrity, survival and function.

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The validity of this theory rests primarily on the demonstration that serum γ-globulin binds to the autologous cell membrane in a specific manner and that in so doing, it affects favorably one or more functions of the cell. To that end, it was shown that only certain fractions of γ-globulin bind tightly and specifically to circulating autologous red cell membrane *in situ* as well as *in vitro* in isotonic media of low ionic strength (2–4). Under these conditions, the bound γ-globulin strengthens the membrane against shearing forces and prevents the rapid fall of the internal ionic concentration of the cell in hypotonic solutions. Further evidence that erythrophilic γ-globulins affect the membrane to influence the shape and conformation of the autologous red cell will be presented later in this report.

In a recent communication, it was shown that dog red blood cells bind certain erythrophilic components of autologous serum γ-globulin. In particular, fraction III as obtained by chromatographic separation on cellulose phosphate (CP) columns was of special interest (2, 5). This fraction is formed primarily by the spleen and its level in the serum is reduced after splenectomy. The decrease in half-life of the red blood cell observed in splenectomized dogs was found to parallel the decrease in this γ-globulin fraction. A considerable improvement of the half-life followed parenteral supplementation which raised the level of fraction III to near normal values. Another, fraction IV binds to a lesser extent but shows no such correlation with the survival of the cell (2).

Human erythrocytes also bind autologous γ-globulin under similar conditions of low ionic strength (4). It was subsequently shown that human red cells and cell membranes also bind specific erythrophilic fractions of autologous γ-globulin in considerable amounts. This is also readily demonstrable *in vitro* in isotonic low ionic strength sucrose medium. Similarly, erythrocyte-bound γ-globulin exists in unaltered samples of circulating blood. The components of erythrophilic γ-globulin correspond primarily to fractions III and IV of serum γ-globulin which are of the γG class. Other minor components, including all isohemagglutinin activity of serum, also bind to erythrocytes under similar conditions and are of the γM and γG class (2, 6).

A similar series of studies demonstrated that a specific leucophilic γ-globulin is bound to the circulating white blood cells of the dog and as such stimulates their phagocytic ability to a considerable extent. This fraction is identical in its physicochemical properties with fraction IV of autologous serum γ-globulin as obtained by chromatography on cellulose phosphate columns (5). *In vitro* binding of this fraction can also be demonstrated in low ionic strength isotonic sucrose medium and can be eluted with 0.15 M NaCl. None of the other fractions (I–III) showed comparably significant binding nor any augmentation of the phagocytic rate. The stimulatory effect of leucophilic γ-globulin on phagocytosis of *Staphylococcus aureus* accounts for almost all that exhibited by autologous serum rendered devoid of any complement or specific antibody (opsonin) activity.

Like the erythrophilic components of autologous γ-globulin, leucophilic γ-globulin fraction IV is also formed in part by the spleen. Its level in serum was significantly reduced in nine out of 15 dogs six to eight weeks after removal of the spleen (2, 6). It has since been found in this laboratory that the serum of such splenectomized animals

or its γ-globulin fraction IV lose their former stimulatory effect on the phagocytic activity of the autologous leucocyte. However, samples of the same cell preparation were stimulated to the fullest extent by the serum or its γ-globulin fraction IV obtained from the same dog prior to the removal of the spleen. It was further found that circulating human leucocytes, like human erythrocytes, bind specific γ-globulin. *In vitro* binding can be readily demonstrated in low ionic strength isotonic sucrose medium.

This type of γ-globulin is of the γG class and has the chromatographic properties on cellulose phosphate columns that correspond to serum γ-globulin fraction IV. A significant portion but not all of the latter binds to leucocytes. This leucophilic portion displays what has been named *leucokinin* activity. This is manifested by its stimulatory effect on the phagocytic function of the autologous polymorphonuclear leucocyte. In the absence of this fraction only minimal phagocytosis for a limited period of time is obtained. In this presentation, I shall review these findings and include additional illustrative data which have recently been obtained in our laboratory.

Materials and Methods

Fresh human or dog blood was obtained with heparin as anticoagulant (25 mg/100 ml). The γ-globulin or its fragments used in all binding and phagocytosis experiments was autologous with respect to the leucocytes and erythrocytes. The various fractions were isolated with column chromatography (5) on cellulose phosphate (CP) (Selectacel, cellulose phosphate, *Carl Scheicher & Schuell Co.*, Keene, N.H.). Columns of 1.5 x 12 cm were used for samples containing 20–25 mg and 0.5 x 12 for samples 4–6 mg of γ-globulin. Protein was measured by optical density at 280 mμ using an extinction of 1.12 or by the *Folin-Lowry* method (7). The preparation of γ-globulin from serum or solutions of its components was done by ammonium sulfate precipitation at 0.33 saturation, then reprecipitation at 0.6 saturation. In all experiments relating to binding of γ-globulin to the cell, the sucrose phosphate solution used for washing was composed of sucrose (0.27 M) in sodium phosphate buffer (5×10^{-3} M, pH 7.4). The NaCl solution was 0.15 M. The media used for phagocytosis were either *Hank*'s medium (8), usually employed for this type of experiment and containing in addition $MgCl_2$ (5×10^{-3} M) at pH 7.4, or our own sucrose medium composed of sucrose (0.27 M), glucose (5.5×10^{-2} M), KCl (1.3×10^{-2} M), $MgCl_2$ (5×10^{-3} M), $CaCl_2$ (5×10^{-4} M), and sodium phosphate buffer (5×10^{-3} M, pH 7.4). In each case, 100 mg of heparin/l of medium was included. All media, solutions and glassware were sterile.

The Phagocytic Reaction. Leucocytes were isolated from the buffy coat. Control samples were employed in all tests. The cells were washed three times with three volumes each of the sucrose medium, separating the leucocyte layer in the process. In this case they remained coated with γ-globulin fraction IV and the phagocytic reaction reached maximal levels. Alternatively, the cells were washed with *Hank*'s medium which is considerably higher in ionic strength. Consequently, the leucophilic γ-globulin was eluted and washed off. Washing was performed three times each with three volumes of the medium and the cells in all cases were sedimented at 250 g. After each washing, the leucocyte layer was separated from the red cells. However, after the third washing the leucocyte layer was almost half of the combined total volume.

The phagocytic reaction (9) was carried out at 37° in siliconed roller tubes with continuous shaking at 8 cycles/min. Coagulase-positive *S. aureus* from an 18 hour culture was added at zero time in a ratio of 1.5 bacteria/polymorphonuclear leucocyte. All protein components added to the reaction mixture were previously dialyzed in the particular medium used in the experiment. All sera and their components used in this study, other than the cellular elements, were heated at 56° for 30 min to destroy complement activity. Because of the possible presence of so-called opsonizing antibody, all samples were absorbed at 37° for 30 min at least with 1.0 mg dry weight of staphylococci/mg of γ-globulin. Further precautions against possible unequal sensitization of the *Staphylococcus organism* by the various samples or fractions, the bacteria were incubated at 30° for 30 min in 1 ml of the particular serum from which the components under study were isolated. The reaction mixture was composed of 0.15 ml of leucocytes suspended in the desired medium containing about 2.0×10^4 cells/cmm mixed with 0.03 ml of the serum component or fraction and allowed to interact for 1 min. *Staphylococcus* culture (0.05 ml) was then added to start the reaction (10, 11).

The rate of the phagocytic reaction was measured by removing a loopful of the mixture at zero time and at other desired periods thereafter for staining and microscopic examination under high power. For proper spreading of the cells on the slide, a loopful of bovine serum albumin, 60 mg/ml was mixed with the sample immediately before smearing. In all cases reported in this study, 200 polymorphonuclear neutrophilic leucocytes were observed. The extent of phagocytosis was recorded as the number of cells containing 1 or more organisms/100 cells. Zero-time samples were removed 15–20 sec after the addition and thorough mixing of the bacteria.

γ-Globulin Fragments. The preparation of Fab and Fc fragments was performed by papain digestion (12) and the $F(ab)_2$ by pepsin digestion (13). These were then isolated by column chromatography on DEAE-Sephadex 50 (11).

Results

I. The Effect of Erythrophilic γ-Globulin on the Autologous Erythrocyte

It is certain that the circulating red cell, like the white blood cell, is coated with specific γ-globulin that has been appropriately termed erythrophilic. The question is whether this γ-globulin coat is important for the integrity, survival and function of the cell. Our results to date indicate that all three of these parameters are affected by the specific coat (2, 3, 6, 11).

The binding of the molecule to the cell membrane occurs at the Fab fragment and not at the Fc. The effects exhibited by the whole molecule are in general duplicated by the isolated Fab or $F(ab)_2$ fragment (11).

A. *The Effect of Erythrophilic γ-Globulin on the Survival of the Erythrocyte*

We have so far conducted studies on two sets of splenectomized dogs with proper sham operated controls (2). Another set of controls were those that were splenectomized and substituted by intravenous treatment with the two erythrophilic fractions isolated

on phosphocellulose columns from pooled dog γ-globulin. All animals that were splenectomized but not treated showed a reduction of the main erythrophilic fraction III to 21–65% of the preoperative level with an average of 42%. Parallelling this was a reduction in the half life of the erythrocyte (2) amounting to 26–50% with an average of 40%. It is noteworthy that dogs that showed the highest reduction of the half life also showed the highest reduction of the level of fraction III. Similarly, animals showing the least reduction of this fraction suffered correspondingly less reduction of the half life. Several months after splenectomy, erythrophilic fraction III reappeared in serum with a parallel return of the half life of the red cell to normal levels.

B. *The Effect of Erythrophilic γ-Globulin on the Red Cell Membrane*

1. A sample of cell membranes coated with the specific γ-globulin and another not coated were suspended in low ionic strength isotonic sucrose 0.27 M and subjected to mild shearing forces. Electron micrographs of these two preparations showed marked differences. The coated membranes retained their general shape and circular contours. By contrast, the uncoated membranes were torn and completely deformed (3). It is reasonable to assume that in this as in the other experiment discussed below, the effect of the autologous γ-globulin is exerted directly on the membrane to which it is bound under conditions of low ionic strength.

2. The permeability of the membrane to ions was studied with the hemolysis technic. It was clear that the γ-globulin coat retards the ionic equilibration between the internal and external environment (3).

3. More impressive results in this connection, have been obtained by observing the effect on the shape of the erythrocyte. The red blood cell is a biconcave circular disc when observed in plasma or serum. It has been known for some time that human red cells, washed with saline, lose the biconcave characteristic, become rounded and exhibit numerous outward projections readily visible with the light microscope. Upon addition of serum, normal shape is immediately resumed (14, 15).

We have observed a similar change in the configuration of the cell (11) also in sucrose solution and shown that the components of serum and plasma that are responsible for this effect are the erythrophilic γ-globulins. Fig. 1 shows the corrugated shape of the erythrocyte in the absence of and the normal shape in the presence of erythrophilic γ-globulin. It takes 30 μg of fraction III, 50 μg of fraction IV and 80 μg of fraction II per ml to effect a 50% reversal of a 2% red cell suspension to normal shapes. All three of these fractions contain erythrophilic γ-globulin. On the other hand, fraction I and serum albumin, neither of which bind to the red cell membrane, require about 200 μg and 300 μg respectively to show a similar effect.

4. In view of the altered shape of the cell and the reduction of the tensile strength of the membrane as observed *in vitro,* as well as the reduction of the half life concomitant with the reduction of the erythrophilic γ-globulin as observed *in vivo* after splenectomy, one might confidently assume that the overall functional activity of the cell would in effect be reduced.

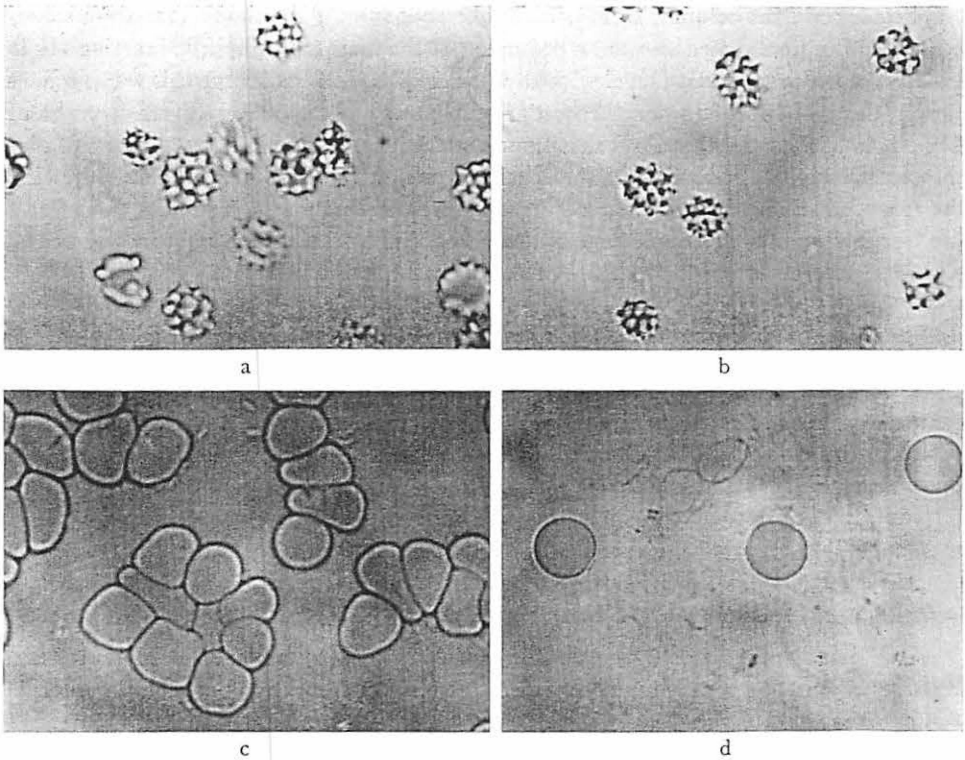

Fig. 1 Shows the difference in conformation of saline washed human erythrocyte in isotonic low ionic strength sucrose medium before and after coating with autologous erythrophilic γ-globulin fraction III. The upper portion of the figure shows the typical crenated appearance of a 2% suspension of cells without a γ-globulin coat. The lower portion shows the same suspension immediately after the cells were coated with erythrophilic γ-globulin fraction III 60 μg per ml (11). See text for details.

II. *The Effect of Leucophilic γ-Globulin on the Phagocytic Activity of the Autologous Polymorphonuclear Leucocyte*

In contrast to the erythrophilic γ-globulin which appears in the three fractions II, III and IV, on phosphocellulose chromatography, the γ-globulin that binds to autologous leucocytes appears only in fraction IV. That portion of fraction IV that binds to the red cell does not exhibit any binding capacity to the white cell and that which binds to the white cell does not bind to the erythrocyte.

Here again the Fab portion of the leucophilic molecule is the fragment responsible for binding to the cell membrane and reproduces more or less the same effects as the parent molecule. Only Fab of fraction IV shows these characteristics. Neither the Fab fragment of other fractions, I-III, nor the Fc of any of the four serum γ-globulin fractions bind or influence the activity of the cell.

It is fortunate that the neutrophilic polymorphonuclear white cell lends itself admirably for functional studies. It exhibits readily measureable phagocytic activity. With

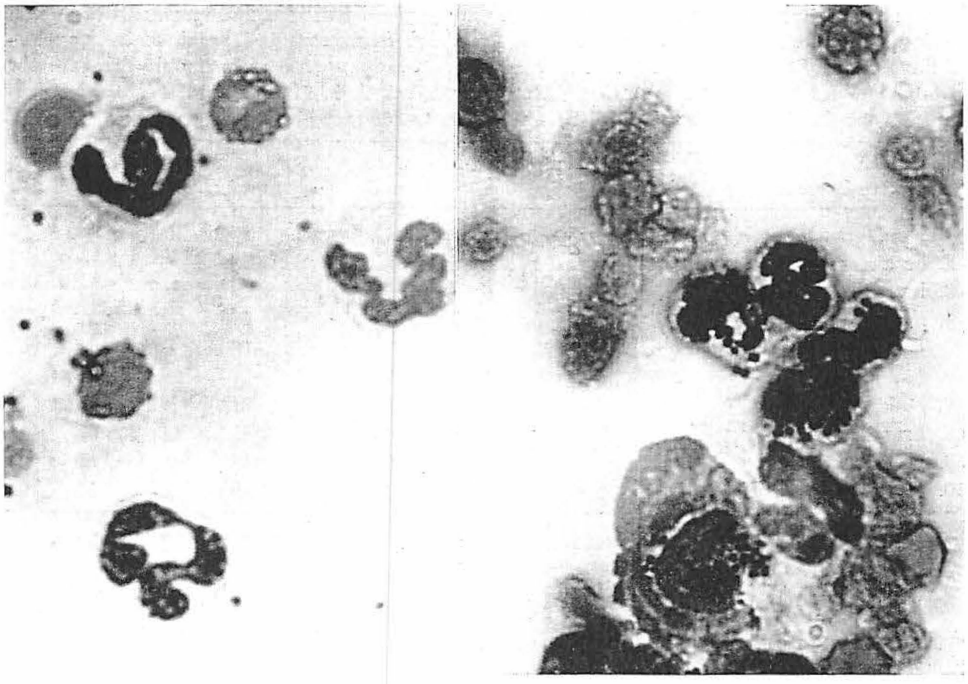

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Fig. 2 Shows the stimulatory effect of leucophilic γ-globulin fraction IV on the phagocytic activity of the polymorphonuclear neutrophile (right) as compared to the lack of effect observed with non-leucophilic fraction I (left). Observe the intracellular localization of staphylococci on the right indicating phagocytic activity as compared to the extracellular localization on the left. See text for details.

this system, we soon were able to show that none of the fractions I-III that fail to bind to the cell affects its phagocytic activity. Only fraction IV and its Fab fragment are capable of this stimulatory effect.

Fig. 2 is illustrative of the typical microscopic field of the phagocytic activity one encounters in the presence of leucophilic fraction IV, as compared to fraction I which does not bind to the membrane. The former stimulates considerable phagocytic activity and most of the bacteria are within the leucocytes. However, with fraction I, little engulfing of bacteria can be observed and most of the bacteria remain in the outside medium.

Table 1 gives comparative and illustrative values of this process. The values, in percent, indicate the number of neutrophiles that have engulfed one or more staphylococci. The stimulatory effect of fraction IV on the phagocytic activity of the polymorphonuclear neutrophile, disappears from this fraction four-six weeks following splenectomy in the dog. It reappears several months later. Whether its reappearance is due to proliferating accessory spleens or to other parts of the lymphatic system is at present unclear.

Table 1 Shows representative values obtained with purified fractions and fragments of human or dog γ-globulin. Leucocytes were obtained from dog buffy coat and prepared as described in the section of Materials and Methods. Fab and Fc were prepared from papain digest of the appropriate fractions and separated on DEAE Sephadex A-50 (Pharmacia). $F(ab)_2$ was prepared by pepsin digestion and separation of Sephadex G-200 (11).

γ-Globulin Fraction or Fragment	Protein μg	% Phagocytosis 10 min.	20 min.	30 min.
1. No addition	–	19	17	18
2. Serum albumin	100	17	19	18
3. Fraction I	100	20	18	17
4. Fraction II	100	19	21	22
5. Fraction III	100	17	19	18
6. Fab fraction I	110	21	22	19
7. Fc fraction I	117	17	21	18
8. Fraction IV	100	30	45	56
9. Fab, Fraction IV	115	23	38	53
10. $F(ab)_2$, fractions II–IV	106	29	40	54

Discussion

The series of approaches reviewed herein for the study of the role of lymphoid system, through its ability to elaborate γ-globulin, have established a new role; a physiological function, that hitherto has not been appreciated. Beyond this immediate role, pertaining to the viability and function of the erythrocyte and polymorphonuclear leucocyte, lie other possible functions that relate to other cellular elements in the circulatory system, including lymphocyte, eosinophile, basophile, platelet and indeed the endothelial cell that lines the vascular channels. These cells by analogy stand out as possible candidates for interaction and association with specific γ-globulins as has already been demonstrated for the erythrocyte and the neutrophile.

One comforting aspect of these findings is the insight it brings to bear on the evolutionary process. It is not necessary to assume any further that nature has fashioned a unique organ of various shapes and localizations for the sole purpose of defense against the invading microorganism. Rather, these organs are strategically placed to synthesize appropriate molecules of γ-globulin that strengthen the membrane of cells that are in constant, physical stress, and influence cell viability and function. It is noteworthy in this connection, that the cells of higher animals are bounded only by the cell membrane. No cell wall is known to exist beyond this membrane. On the other hand, the plant and the bacterial cell have, in addition to the membrane, a cell wall of elaborate layers of polymeric substances such as cellulose, protein, mucopeptide, and teichoic acid, as well as capsular material etc. One may ask whether the binding of γ-globulin to the membrane of these cells, though noncovalent, may not also play role similar to the walls of lower forms of life in lending strength and exerting control on the flow of ions and small molecules across the membrane. In addition, these γ-globulin molecules may be carriers, covalently or by association, of active substances that can be brought to the immediate proximity of the membrane to influence its activity and thus the function of the cell.

The evolution of the antibody, as a γ-globulin variant of the physiologically active molecule, endows the animal with a defense mechanism of considerable survival value. There would reasonably follow a selective evolutionary advantage and a further strengthening of the defense mechanism. It is such a variant, of epidermal hair, bunched together on the nose of a rhinoceros, that finally culminated in its present and effective nose horn.

Summary

A physiological role of γ-globulin has been established for human and dog erythrocytes and polymorphonuclear leucocytes. Specific γ-globulin binds to the membrane of the cells and determines their conformation, viability and function. The spleen appears to be primarily responsible for the synthesis and excretion of these γ-globulin molecules.

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Blood-Borne Virus-Like Agents in Hodgkin's Disease, other Malignancies, and Systemic Lupus Erythematosus

Results of a Large Scale Survey by Means of a New Serological Screening Test

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The studies reported here embody a new principle in technical methods for detection of hidden viral infection as applied to certain disease categories. The technique is based on the long known observation that the red blood cell can adsorb to its surface a large variety of viruses (1) and virus-like agents and may thereby become the vehicle of their transport in the circulating blood (2). Red blood cells circulating innumerable times through diseased tissue may be held to pick up with each successive passage an additional "load" of viral material. An illustrative parallel may be drawn with the selective affinity of certain ion-exchange reins for specific non-living molecules. Repeated perfusion of the tissue with the same blood cells multiplies their chances of accumulating a rela-

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