*THE PHYSIOLOGICAL ROLE OF THE LYMPHOID SYSTEM, III. LEUCOPHILIC γ-GLOBULIN AND THE PHAGOCYTIC ACTIVITY OF THE POLYMORPHONUCLEAR LEUCOCYTE**

By B. V. Fidalgo and V. A. Najjar

DEPARTMENT OF MICROBIOLOGY, VANDERBILT UNIVERSITY SCHOOL OF MEDICINE, NASHVILLE, TENNESSEE

Communicated by Earl W. Sutherland, Jr., January 16, 1967

During the past decade a series of investigations in this laboratory, dealing with the mechanism of antibody-antigen interaction, led to a new concept proposed by Najjar in 1963:[1] that the lymphoid system plays a physiological role with the primary purpose of producing specific γ-globulins that bind to complementary receptor sites on the cellular membrane. These proteins are presumed to be necessary for its structural integrity and function, and therefore for the physiology and survival of the cell. The elaboration of antibody by the same lymphoid tissue is nevertheless an important major function and would be an expression of essentially the same phenomenon in response to the intrusion of an unfamiliar and antigenic molecule.[1-3] In this respect, this phenomenon would be similar to the detoxification function of the liver: for example, acetylation, methylation, glucuronidation, sulfation, etc. At one time these were believed to be specialized functions that neutralized extraneous toxic amines, phenols, alcohols, etc. All have since been recognized as manifestations of essential biochemical reactions ordinarily engaged in the normal metabolic process and, like antibody formation, serve an important defensive function. This and a recent report[4] present evidence in favor of the theory that specific γ-globulins play a physiological role essential to the normal function of the cellular elements of the blood, the leucocyte, and the erythrocyte.

The validity of this theory rests primarily on (*a*) the demonstration that serum γ-globulin binds to the autologous cell membrane in a specific manner and (*b*) 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 autologous red cell membrane *in situ* and under isotonic conditions of low ionic strength.[4-6] 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 media.[5]

This report is concerned with a particular fraction, leucophilic γ-globulin fraction IV, which is isolated by cellulose phosphate (CP) chromatography, which binds specifically to autologous blood leucocytes, and stimulates phagocytosis of *Staphylococcus aureus* almost to the full extent observed in normal autologous serum. An earlier communication that appeared in a previous issue of this journal was concerned with an erythrophilic γ-globulin, primarily CP fraction III, which binds specifically to red blood cells and appears to be necessary for the normal survival of the cell.[4]

Materials and Methods.—The various serum and cell components were obtained from normal healthy mongrel dogs of both sexes weighing 12–15 kg. As anticoagulant, citrate and glucose were added at a final concentration of 1.6×10^{-2} *M* each at pH 7.4. Saline (0.15 *N* NaCl) was used unbuffered. Hank's solution (medium)[7] was used throughout with $MgCl_2$ 5×10^{-3} *M* at pH 7.4. The sucrose solution (medium), like Hank's medium, contained 100 mg of heparin per

liter and was composed of sucrose 0.27 *M*, glucose 0.055 *M*, sodium phosphate buffer 0.005 *M*, potassium chloride 0.013 *M*, and magnesium chloride 0.005 *M*, pH 7.4. Serum and cell γ-globulin fractions used in this study were completely soluble in this medium.

The fractionation procedure: The fractionation of γ-globulin from serum followed essentially the same procedure devised for the separation of human γ-globulin fractions.[8] Plasma was converted to serum by clotting in calcium chloride 2.5×10^{-2} *M* final concentration. γ-Globulin was precipitated twice in 33% ammonium sulfate, dialyzed against acetate buffer 0.05 *M*, pH 4.8, and applied to a cellulose phosphate (CP) column pre-equilibrated with the same buffer. Fractions I, II, and III were eluted with 0.15 *N* NaCl in 0.05 *M* acetate buffer at pH 4.8, 5.0, and 5.2, respectively. Fraction IV was then eluted with 0.2 *M* NaCl in the same buffer but at pH 5.4. The fractionation pattern is shown in Figure 1. It is essentially similar to that obtained with human γ-globulin except that the relative proportions of the fraction vary in a manner characteristic of this species. Dialysis was performed in the cold room.

Phagocytosis reaction: In all phagocytosis experiments an 18-hr culture of a coagulase-positive *Staphylococcus aureus* was used. Leucocytes were isolated from the buffy coat and washed either (*a*) with the sucrose medium, in which case they remained coated with fraction IV (coated cells), or (*b*) with Hank's medium, in which case they were stripped of the γ-globulin coat (naked cells). Washing was performed with 2.5 vol three times and sedimented at 250 × *g*. This procedure served to separate further the leucocyte layer from the contaminating red cells. However, the final leucocyte suspension used in all experiments on phagocytosis contained about 40% leucocytes and 60% red cells v/v. Each of the various serum components and controls was heated at 56° for 30 min to destroy any possible complement activity. Because of the possible presence of opsonizing antibody, the sample was absorbed three times at 37°C for 30 min each with 1.0 mg (dry weight) of staphylococci per mg of γ-globulin. Further precautions against possible unequal sensitization of the test organisms by the various components, staphylococci were incubated at 30°C for 30 min in 1 ml of the same fresh dog serum from which the components under investigation were prepared. When these precautions were omitted, no detectable effects on the rate of phagocytosis were observed, an indication that the sera studied did not possess any opsonizing antibody against the organisms.

The procedure used for phagocytosis was an adaptation of that described by Rogers and Melly.[9] It was carried out in siliconed roller tubes at 37°C with continuous shaking at 8 cpm. The protein components of the reaction mixture were dialyzed, and the cellular components washed and suspended in the sucrose medium. When phagocytosis was studied in Hank's medium, the latter was used for the dialysis and washing instead. To 0.3 ml of the thrice-washed leucocyte suspension containing 23,000–28,000 cells per cm, 0.05 ml of the serum component or fraction containing the desired quantity of protein was added and incubated for 10 min. This permitted early interaction, where possible, between the protein and the cell membrane. Finally, 0.05 ml of a suspension of coagulase-positive *Staphylococcus aureus* prepared from an 18-hr culture was added at a ratio of 1.5 organisms per polymorphonuclear cell. Incubation was continued for a maximum of 60 min at which time samples were obtained at chosen intervals for staining. The extent of phagocytosis was evaluated by microscopic scanning under high power and recorded as the number of leucocytes containing one or more staphylococci per 100 cells. In each experiment 200 cells were observed. The average number of bacteria engulfed per leucocyte in general paralleled the extent of phagocytosis.

Results.—Leucocyte-bound γ-globulin: It was possible to demonstrate that dextran-isolated blood leucocytes[10] that are washed thoroughly with an enriched low-ionic-strength sucrose medium (see below), like red cells,[5,6] also retain tightly bound γ-globulin. In like manner, this is readily eluted from the cells with solutions of higher ionic strengths such as 0.15 *N* NaCl or Hank's solution containing isotonic salt concentration. The amount of fraction IV eluted was found to be 1–1.2 mg of protein/ml of leucocytes packed at 490 × *g* for five minutes. Chromatographic properties of the desalted globulin on CP columns are identical with those of fraction IV isolated from serum γ-globulin. Naked leucocytes, stripped of γ-globulin by washing with Hank's solution or saline, bind only fraction IV in the

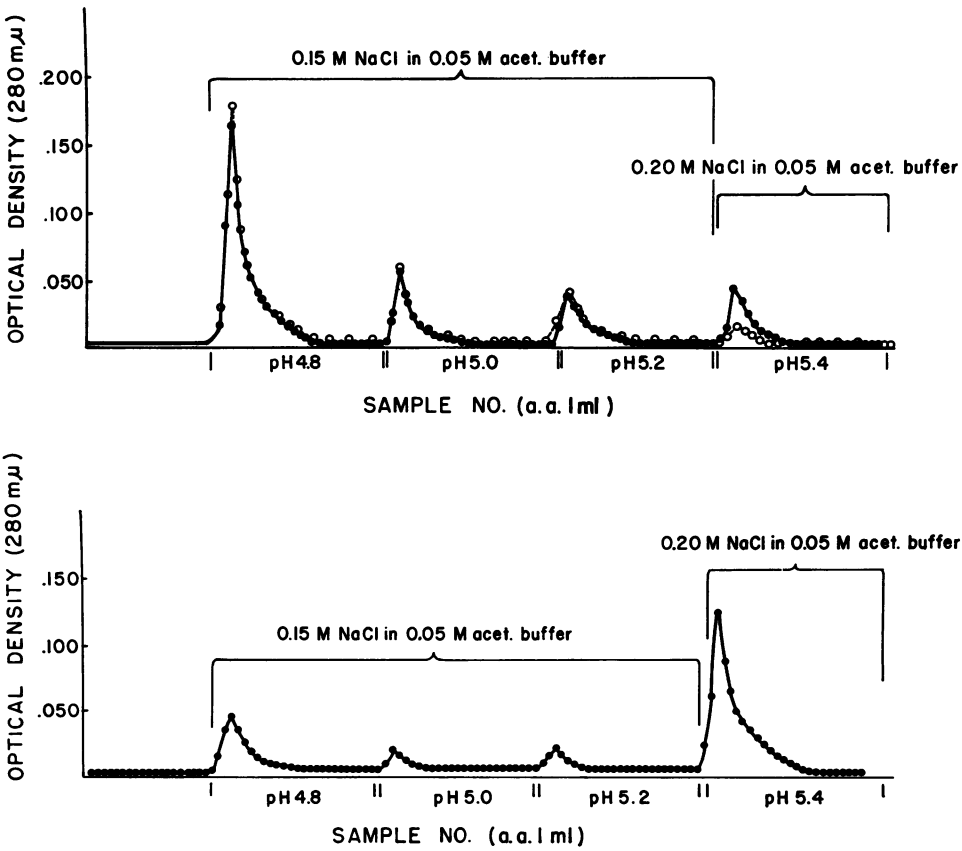

FIG. 1.—Shows that naked leucocytes bind only γ-globulin IV from whole serum γ-globulin causing a reaction in the fraction. Whole γ-globulin was dialyzed against sucrose solution. Five milliliters containing 50 mg were absorbed for 20 min at 4°C with 6 ml of packed naked leucocytes previously purified through dextran[10] and washed with saline, then sucrose solution. After centrifugation, the supernatant was removed and the sedimented cells were then eluted twice with 2.5 vol of saline each. The eluate was then concentrated to 2 ml by evaporation in a dialysis bag in the cold room. This was followed by dialysis against 0.05 *M* acetate buffer pH 4.8. Aliquots containing 5 mg each of (●) whole γ-globulin before absorption, (○) after absorption (*upper graph*), and γ-globulin eluted from the leucocytes (*lower graph*) were chromatographed on 10 × 0.5-cm CP column. The leucocyte preparation contained a negligible volume of contaminating red cells (30% by count). The erythrocyte-bound globulin would not be detectable in this experiment, since it would contribute about 0.8 mg per ml only. Such preparation of leucocytes yielded 1–1.2 mg of γ-globulin/ml of cells packed at 490 × *g*.

sucrose medium containing whole γ-globulin. This occurs readily at room temperature or 0°C. Figure 1 shows the specific reduction in fraction IV in the supernatant after absorption of whole γ-globulin with naked white cells as compared to the pattern obtained before absorption. The eluted γ-globulin from the leucocytes shows the predominance of fraction IV, with minor components of the other fractions. This pattern has been repeatedly observed. It is unlikely that the minor components are simple contaminants and may well be specifically bound to the cell membrane much as fraction IV but in much smaller quantities.

Leucocyte-bound γ-globulin in situ: Since leucocytes normally survive and function in plasma, it is of considerable interest to determine whether in this en-

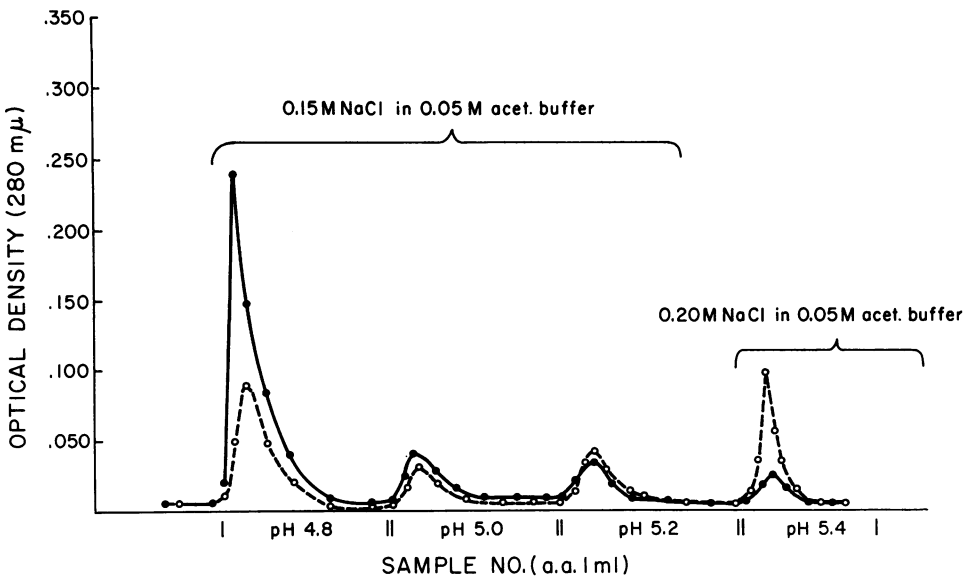

FIG. 2.—Shows the presence of leucophilic γ-globulin *in situ*. The pattern (○) of the eluate from isolated leucocytes of the buffy coat (see text) shows a preponderance of fraction IV, as compared with the pattern (●) of whole γ-globulin. Citrated blood (300 ml) was centrifuged at 490 × g for 5 min. The buffy coat was removed along with plasma and resedimented three more times to separate further the contaminating red cells. The supernatant plasma was sucked off gently. The cells and the remaining plasma were then washed twice with 2.5 vol each of saline. The pooled washings were then chromatographed (for details see Fig. 1 and text).

vironment these cells are in fact coated with γ-globulin. If such is the case, it would be necessary to determine whether the bound γ-globulin is the same fraction IV isolated after manipulative washing with an unphysiological though isotonic sucrose solution. Accordingly, the buffy coat was isolated by sedimenting the red cells from 300 ml of blood. The white cells were then repeatedly sedimented in their own plasma four to six times. Each time, the leucocyte layer was carefully removed to effect a separation from red cells. The plasma-packed leucocytes were then washed two times with saline 2.5 volumes each. The pooled washings were dialyzed against sodium acetate buffer 0.05 *M*, pH 4.8. This was then applied to the CP column and chromatographed. The fractionation pattern is shown in Figure 2, superimposed on the pattern obtained of the serum γ-globulin. With this procedure it would be expected that the saline washings of the packed leucocytes would contain all the four fractions of the γ-globulin present in the contaminating plasma as well as that portion of the γ-globulin that might be bound to the leucocytes. Whatever that fraction might be, it would be reflected by a relative increase in the corresponding plasma γ-globulin fraction. It is apparent from the figure that of all the fractions, only fraction IV was augmented. This is a clear indication that in plasma the leucocytes do retain a considerable amount of bound fraction IV. One cannot escape the conclusion that these cells circulate, survive, and function in the blood with a coat of γ-globulin forming an intimate part of its outer structure.

The identity of leucophilic γ-globulin with fraction IV: γ-Globulin eluted from leucocytes has the following characteristics in common with serum γ-globulin

fraction IV: (*a*) precipitability in 0.33 ammonium sulfate saturation, (*b*) mobility on paper electrophoresis and immunoelectrophoresis on agar gel, (*c*) chromatographic properties on cellulose phosphate columns, and (*d*) binding capacity to leucocytes. (*e*) Furthermore, as will be shown below, its capacity to stimulate phagocytosis of *Staphylococcus aureus* is also similar to fraction IV obtained from serum γ-globulin. (*f*) Immunochemically, like fraction IV, it belongs to the γG class of γ-globulin, and immunodiffusion on agar gel shows the characteristics of identity to fraction IV with merging of the lines as shown in Figure 3. In view of this, it can be safely assumed that the γ-globulin coat on the leucocyte derives from fraction IV of serum γ-globulin.

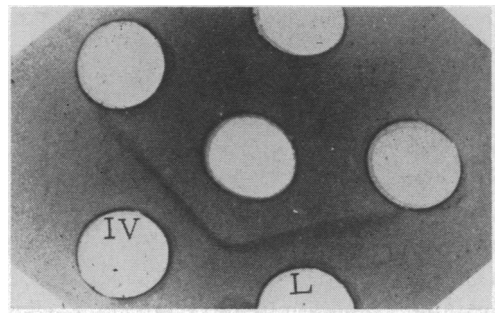

FIG. 3.—Shows the identity of the leucophilic γ-globulin bound to the white cells, with fraction IV of whole γ-globulin. Leucophilic γ-globulin (*L*) was eluted from leucocytes, concentrated, and dialyzed against saline. Fraction IV was prepared as usual, and 100 μg of each was run in an agar diffusion plate against rabbit anti-canine serum (Hyland Lab). (For details see Fig. 1 and text.)

The stimulatory effect of added fraction IV on phagocytosis: Based on the reasoning that the presence of cell-bound specific γ-globulin is purposeful and therefore important for cell function and survival, experiments were carried out that were designed to test this hypothesis. A number of parameters are currently being tested, including mobility of the leucocyte, its survival, and metabolic activity. One of the most sensitive parameters is phagocytosis. It represents an important functional aspect that encompasses most if not all of the other parameters. Consequently, various components of serum were tested for their capacity to stimulate phagocytosis in the sucrose medium: (*a*) whole serum, (*b*) whole γ-globulin, (*c*) the supernatant fraction from a 50 per cent ammonium sulfate cut of serum containing, among other components, albumin and α and β globulins, (*d*) the individual serum γ-globulin fractions I to IV, (*e*) fraction IV isolated from leucocytes, (*f*) purified dog serum albumin, and finally, bovine and rabbit serum albumin which

TABLE 1

STIMULATION OF PHAGOCYTOSIS BY LEUCOPHILIC FRACTION IV

Autologous components added per ml of packed leucocytes		Extent of Phagocytosis in Sucrose Medium after 30 Min		
		(μg)	Dog M17 (%)	Dog M18 (%)
Albumin		500	16	20
γ-Globulin fraction	I	"	15	18
" "	II	"	20	10
" "	III	"	16	12
" "	IV	"	45	50
" "	IV	250	43	42
γ-Globulin eluted from sucrose-washed leucocytes		"	46	43
Whole γ-globulin		500	36	39
Serum, 0.18 ml, or 0.2 ml containing fraction IV		"	53	49
Serum, 0.09 ml, or 0.1 ml containing fraction IV		250	51	47
No addition			1	3

Table 1 shows the effect of leucophilic γ-globulin fraction IV on phagocytosis which is comparable to that obtained by whole serum containing similar quantities of the fraction. Values are to the nearest per cent. The quantities of γ-globulin shown refer to the amount calculated per ml of leucocytes packed at 250 × *g* for 5 min and containing about 60% red cells by volume. Full details are given in the text.

served as nonspecific controls. The cells tested were naked leucocytes isolated by washing in Hank's medium. The results are shown in Table 1. These include minimal amounts of fraction IV that gave maximum phagocytosis, and indicate that fraction IV, whether derived from serum γ-globulin or from leucocytes, is the only component that approaches the stimulatory effects on phagocytosis exerted by whole serum. The latter was added in quantities that contained comparable amounts of fraction IV. The maximum phagocytosis of 45–50 per cent obtained in these experiments could not be exceeded under the circumstances of the experiments since at this level of phagocytosis, little or no bacteria remained free in the medium. The stimulatory effect of leucophilic γ-globulin fraction IV on phagocytosis, shown in these experiments, parallels its unique property of specific binding to the phagocytes in our low ionic sucrose medium. Accordingly, the effect must have been exerted by those molecules that are bound to the membrane of the cells. This conclusion is verified and reinforced by the demonstration that full phagocytic activity is invariably exerted by cells that are isolated in the coated state. This is accomplished by isolating the buffy coat in the sucrose medium.

The phagocytic capacity of leucocytes isolated in the coated state: This type of experiment is rather unique, as compared to the usual studies on phagocytosis. It has been the accepted custom to add serum to the enriched isotonic salt medium, since it was found to be necessary for the process. However, under the conditions of this experiment, maximum phagocytosis occurred when washed leucocytes were allowed to engulf bacteria in the sucrose medium in the absence of added serum. The cells were prepared by washing the buffy coat with the sucrose medium. Under these conditions, the leucocytes retain their natural *in situ* coat of leucophilic γ-globulin which otherwise would be lost in the usual isolation procedure involving washing with Hank's medium. Furthermore, phagocytosis was carried out in the low ionic strength medium in which the dissociation equilibrium is heavily in favor of binding. The data are represtented in Table 2 and show that maximal phagocytosis is obtained with the coated phagocytes. Those not coated are capable only of minimal phagocytosis comparable to that obtained in Hank's medium under similar conditions. Figure 4 shows a microscopic view of a representative field observed in

TABLE 2

PHAGOCYTIC ACTIVITY OF LEUCOPHILIC γ-GLOBULIN COATED POLYMORPHONUCLEAR CELLS

	Extent of Phagocytosis in Sucrose Medium after 30 Min	
	Naked leucocytes (washed with Hank's medium)	Coated leucocytes (washed with sucrose medium)
Dog No.	(%)	(%)
MP 21	3	55
MP 22	1	43
MP 23	2	45
MP 24	1	52
MP 17	7	49
MP 18	3	50
MP 19	4	42
	Plus fr. IV and albumin	Plus albumin
MP 17	44	51
MP 18	46	55
MP 19	47	48

Table 2 depicts the maximal efficiency shown by leucocytes isolated under conditions where they are already coated with leucophilic γ-globulin. Maximal phagocytosis occurred in sucrose medium without any additions, as compared with added γ-globulin fraction IV, 250 μg per ml of cells packed at 250 × *g* and containing 60% red cells, albumin 250 μg. Values are to the nearest per cent. Full details are given in the text.

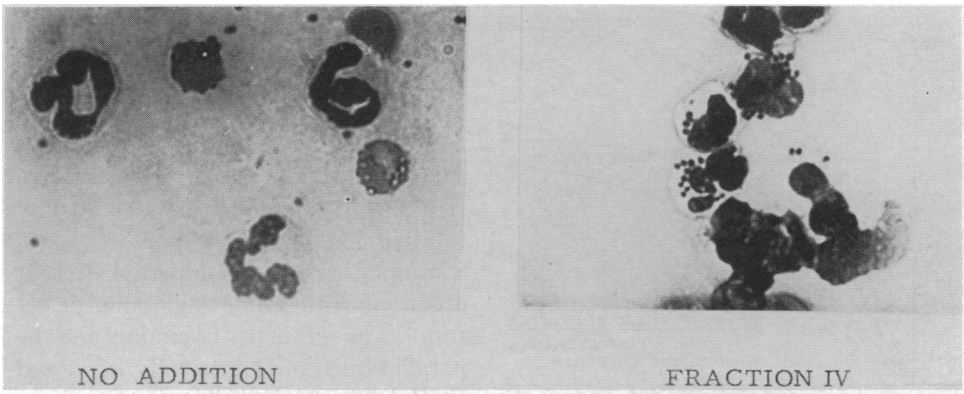

FIG. 4.—Shows the high extent of phagocytosis of leucophilic γ-globulin-coated cells as compared to uncoated cells. Phagocytosis was carried out in the sucrose medium. The smears shown above were taken at 30 min (for details see text).

these experiments. It illustrates that coated cells engulf staphylococci with considerable efficiency, leaving little or no free bacteria in the medium. By contrast, naked cells show only sporadic phagocytosis. Consequently, a considerable number of bacteria are present in the medium.

The kinetics of phagocytosis with leucophilic γ-globulin fraction IV: The rate of phagocytosis in the presence of 250 μg of fraction IV/ml of packed naked leucocytes was compared with that obtained with whole serum 0.1 ml, which contained the same amounts of the fraction. This was done in the sucrose medium as well as in Hank's medium. The purpose was twofold: (*a*) to compare the efficiency of the specific fraction with that exerted by serum, neither of which contains opsonins or complement activity; and (*b*) to compare the two media, one of which, the sucrose medium, favors maximal binding of the γ-globulin, and the other, Hank's medium, lowers the binding capacity by virtue of the higher ionic strength. Figure 5 shows that with both γ-globulin and serum our medium is considerably more conducive to efficient phagocytosis than Hank's medium. To obtain a comparable level of phagocytosis in Hank's medium, it was necessary to add at least five times the quantities used of serum and fraction IV. This was not an unexpected finding and emphasizes that the rate of phagocytosis bears a direct relationship to the amount of bound γ-globulin. Because the binding is ionic in character, an increase in the ionic strength of the medium would require larger concentrations of the fraction to obtain comparable amounts of cell-bound globulin. The figure also shows that a comparable amount of fraction IV is slightly though consistently more efficient in its native state in serum than when isolated by the techniques used in this study. This could be the result of partial denaturation of fraction IV during isolation. It is noteworthy that the minimum amount of leucophilic γ-globulin that effects maximum phagocytosis is within 30 per cent of the amount that the leucocyte is capable of binding.

Discussion.—Taken together, these findings strongly indicate that leucophilic γ-globulin exerts a physiological function that is important for the phagocytic activity of the cell. A number of related observations favor this conclusion: leucophilic γ-globulin is bound to the surface of the cell *in situ*. It is identical, in

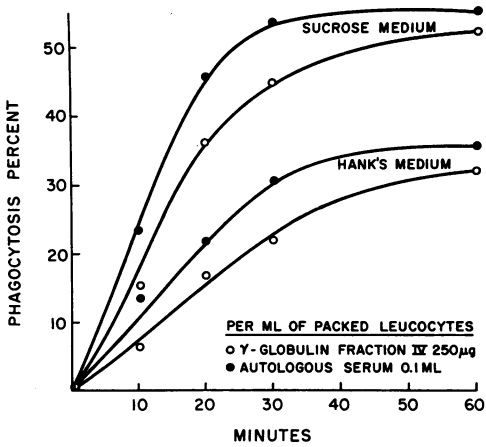

FIG. 5.—Shows the relative stimulatory effects on phagocytosis of isolated γ-globulin fraction IV as compared to serum containing the same amount of the fraction. It also emphasizes the advantages of our sucrose medium over Hank's medium (for details see text).

all parameters tested, with γ-globulin fraction IV present in the plasma. Leucocytes isolated from plasma by washing with low ionic strength solutions retain only this fraction bound in substantial quantities to their surface structure. Naked leucocytes bind only fraction IV. Only fraction IV exerts a stimulatory effect comparable to that observed with whole γ-globulin or serum. The specificity of binding and the parallel functional specificity expressed in phagocytosis support this concept. It is reasonable to assume that the binding observed *in situ* must also exist in the circulating plasma *in vivo*. Consequently, it can be inferred that leucophilic γ-globulin plays a similar role in augmenting phagocytosis *in vivo* in the blood and perhaps in tissues as well.

Conclusion.—Dog leucocytes bind a specific type of γ-globulin *in situ*. This is identical with fraction IV of serum γ-globulin isolated by cellulose phosphate chromatography. This fraction stimulates phagocytosis *in vitro* to levels comparable to that obtained for complement-inactivated normal serum. A medium of low ionic strength is described that is considerably more suitable for this type of investigation on phagocytosis than Hank's medium.

We are indebted to E. Stitt for technical assistance and to M. A. Melly for a helpful demonstration of the phagocytosis technic.

* This investigation was supported by a grant (AI-06191) from the National Institutes of Health, U.S. Public Health Service.

[1] Najjar, V. A., *Phys. Rev.*, **43**, 243 (1963).

[2] Harshman, S., J. P. Robinson, and V. A. Najjar, *N. Y. Acad. Sci.*, **103**, 688 (1963).

[3] Harshman, S., V. A. Najjar, *Am. J. Hyg.*, Monographic Series, no. 22 (1963), p. 20.

[4] Fidalgo, B. V., V. A. Najjar, C. F. Zukoski, and Y. Katayama, these PROCEEDINGS, **57**, 665 (1967).

[5] Najjar, V. A., J. P. Robinson, A. R. Lawton, and B. V. Fidalgo, *Johns Hopkins Med. J.*, **120**, 63 (1967).

[6] Harshman, S., and V. A. Najjar, *Biochem. Biophys. Res. Commun.*, **11**, 411 (1963).

[7] McKinney, G. R., S. P. Martin, R. W. Rundles, and R. Green, *J. Applied Physiol.*, **5**, 335 (1953).

[8] Thomaidis, T., B. V. Fidalgo, S. Harshman, and V. A. Najjar, in preparation.

[9] Rogers, D. E., and M. A. Melly, *Yale J. Biol. Med.*, **34**, 560 (1962).

[10] Skoog, W. A., and W. S. Beck, *Blood*, **11**, 436 (1956).