The Physiological Role of the Lymphoid System. VII. The Disappearance of Leucokinin Activity Following Splenectomy*

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ABSTRACT: Leucokinin, the specific γ-globulin that coats the blood leucocytes and is necessary for its phagocytic activity, becomes inactive following splenectomy. It is devoid of any stimulatory effect on phagocytosis. However, the leucocyte is unimpaired. It exhibits maximal stimulation when coated with active leucokinin obtained before removal of the spleen. The level of erythrophilic γ-globulin cellulose phosphate fraction III in serum is considerably reduced with a parallel reduction of the half-life of the red cell.

This is in agreement with a similar study reported earlier. Supplementation of both γ-globulins by weekly intramuscular injections results in the complete maintenance of normal leucokinin activity, normal level of erythrophilic γ-globulin, and normal erythrocyte half-life.

The physiological role of γ-globulin proposed earlier (Najjar, 1963) has been supported by recent findings. It was shown that in the blood of man and dog, the erythrocytes and leucocytes possess membrane-bound γ-globulin coats. The type of γ-globulin is quite specific to the particular cell. The erythrophilic γ-globulin which binds to the membrane of the red cell is necessary for the integrity and normal survival of the cell (Najjar *et al.*, 1967; Fidalgo *et al.*, 1967a,b). It has not been determined whether leucophilic γ-globulin that binds to the membrane of the white blood cell, is necessary for its normal survival. However, it was shown that the bound γ-globulin, which has been termed leucokinin, is necessary for the attainment of maximum *in vitro* phagocytic activity of the polymorphonuclear leucocyte of the dog (Fidalgo and Najjar, 1967a) and man (Fidalgo and Najjar, 1967b).

Within 4–8 weeks following splenectomy in the dog, the main serum γ-globulin fraction that binds to the erythrocytes, erythrophilic γ-globulin, is considerably decreased. Concomitantly, there is also a marked reduction of the half-life of the red cell reaching, in some instances, a level about 50% of the normal value. Several months after splenectomy, there follows a gradual increase of this fraction approaching normal values between the 4th and 8th month. At the same time, the half-life of the red cell also approaches normal values. The main purpose of this paper is to show that 4–8 weeks following splenectomy, the stimulatory activity of leucokinin on the phagocytic ability of the leucocyte virtually disappears. The leucokinin activity finally reappears and reaches normal values several months after splenectomy. The levels of the erythrophilic γ-globulin and the half-life of the erythrocyte were also followed. The results confirm the previous findings of a parallel reduction of this fraction of γ-globulin and of the erythrocyte half-life with subsequent simultaneous recovery.

Materials and Methods

Mongrel dogs of both sexes weighing 18–25 kg were employed. Splenectomy was performed in the usual manner under strict surgical technic. Blood cultures for Bartonella were taken routinely one to two times monthly and prophylactic procaine penicillin 1×10^6 units was given intramuscularly once weekly as a prophylactic measure against infection. No infection of any sort was detected at any time and all animals remain healthy to date, over 1 year after the removal of the spleen. Blood was withdrawn from the femoral vein into heparin, 15 mg/100 ml. Fractionation of γ-globulin on cellulose phosphate (CP)[1] (Selectacel CP, Carl Schleider & Schuell Co., Keene, N. H.) columns into four fractions I–IV was performed as before (Thomaidis *et al.*, 1967). The half-life of the red cell was measured by ^{51}Cr tagging (Waldman *et al.*, 1960). The phagocytic ability of the polymorphonuclear leucocyte was assayed under sterile conditions with sterile solutions and glassware (Fidalgo and Najjar, 1967a,b). The media used were either Hank's (McKinney *et al.*, 1953) with added magnesium chloride (5×10^{-3} M) or low ionic strength isotonic sucrose medium (Fidalgo and Najjar, 1967b), as the case may be. In all instances, where minimal

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CP, cellulose phosphate; γG, γ-globulin.

amounts of serum or CP fraction IV were used that yield maximal rates of phagocytic activity in the sucrose medium, Hank's medium again proved far inferior for this purpose, as was shown earlier (Fidalgo and Najjar, 1967a,b). The sucrose medium was composed of sucrose (0.27 M), glucose (5.5×10^{-2} M), potassium chloride (1.3×10^{-3} M), magnesium chloride (5×10^{-3} M), and calcium chloride (5×10^{-4} M) in sodium phosphate buffer (5×10^{-3} M, pH 7.4). In all experiments an 18-hr culture of coagulase-positive *Staphylococcus aureus* was used. Serum or its various γ-globulin fractions were heated at 56° for 30 min to destroy complement activity. These were then absorbed at 37° with 1.7 mg dry weight of the bacteria/mg of γ-globulin in the sample. All samples of the staphylococci used for phagocytosis were preincubated for 1 hr at 37° with 1 ml of dog serum obtained before the splenectomy. All serum samples or γ-globulin fractions, as well as the blood cells, were autologous with respect to one another in any one experiment. Fresh leucocytes were obtained from the buffy coat immediately before use. These were washed four times at room temperature with three volumes each, either with Hank's medium to yield uncoated, naked cells (Fidalgo and Najjar, 1967a), or with our sucrose medium, in which case the cells retained their γ-globulin coat, coated cells. They were further washed once with the particular medium used for phagocytosis. The final concentration of leucocytes was adjusted to $1.8–2 \times 10^7$ cells per ml. The reaction mixture was composed of: (a) leucocytes preparation (0.3 ml), (b) serum or serum fractions (0.05 ml), and (c) staphylococci (0.05 ml) such that a ratio of 1.5 bacteria/white blood corpuscle was obtained. Normal serum (0.03 ml) contained approximately 0.1 mg of CP fraction IV and represented the minimum amount required for maximum stimulation of the phagocytic rate. The reaction was carried out in silicone-coated roller tubes at 37° with continuous shaking at 8 cycles/min in a vertical circular rotor. The protein components were dialyzed and the cellular components were washed and suspended in the same medium used for phagocytosis. Phagocytosis was assayed as usual by microscopic observation under high-power oil immersion. The number of neutrophilic polymorphonuclear leucocytes showing one or more engulfed staphylococci per 100 cells represented the extent of the phagocytic activity. A total of 200 cells were counted for each determination. Eosinophils and basophils were excluded from the count. Samples were taken at 0, 5, 10, 20, and 30 min after the addition of bacteria. Further details of the procedure were as described before (Fidalgo and Najjar, 1967b). Protein measurements were made according to Lowry *et al.* (1951).

Results

Six dogs were splenectomized. Two of these (21 and 30) were given weekly injections of 250 mg each of CP fractions III and IV. Two sham-operated dogs served as controls. The quantitative pattern of the various fractions of γ-globulin were studied by CP chromatography (Thomaidis *et al.*, 1967) before and after splenectomy. The results were similar to those observed earlier (Fidalgo *et al.*, 1967b). There was a marked reduction of the erythrophilic fraction III in all animals except the nonsplenectomized or treated controls. The leucophilic CP fraction IV, however, showed definite reduction only in one of the splenectomized untreated animals. This amounted to 38% of its respective presplenectomy value. Figure 1 illustrates the pattern of the four fractions in the dog showing reduction of both CP fractions III and IV.

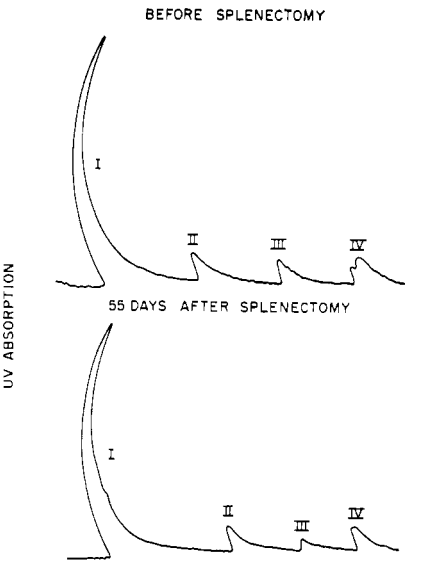

FIGURE 1: The reduction of CP fractions III and IV following the removal of the spleen. γ-Globulin (20 mg) was fractionated on a 10×1.5 cm cellulose phosphate column previously equilibrated in sodium acetate buffer (0.05 M, pH 4.8). Fractions I–III were eluted with 0.15 M NaCl in the same buffer at pH 4.8, 5.0, and 5.2, respectively. Fraction IV was eluted with 0.2 M NaCl also in 0.05 M acetate buffer, at pH 5.4. γ-Globulin was obtained by two precipitations in ammonium sulfate 0.33 saturation and dialyzed against acetate buffer (0.05 M, pH 4.8) (Thomaidis *et al.*, 1967).

The Reduction in Leucokinin Activity. It was shown previously that only the leucophilic component of CP fraction IV binds to leucocytes (Fidalgo and Najjar, 1967a,b). Another portion of this fraction specifically binds to red blood cells and constitutes the minor component of the specific erythrophilic γ-globulin (Fidalgo *et al.*, 1967a,b). The third portion does not bind to either cell. Only the leucophilic component of this fraction, leucokinin, stimulates the phagocytic activity of the neutrophilic polymorphonuclear leucocytes. The nonsplenectomized animals exhibited no reduction of leucokinin activity. Similarly, the two animals treated by weekly injections of both CP fractions III and IV also showed no reduction of this activity. On the other hand, the four splenectomized but untreated animals lost all leucokinin activity as tested either with whole serum, with isolated γ-globulin CP fraction IV, or with the leucophilic component of fraction IV.

In all these experiments, naked leucocytes were used to measure the stimulation of the phagocytic rate exerted by serum and CP fraction IV. However, for the assays of the stimulating effect of already bound leucophilic component of fraction IV, the cells were washed with low ionic strength sucrose medium, in which case they remained coated with the specific γ-globulin. The

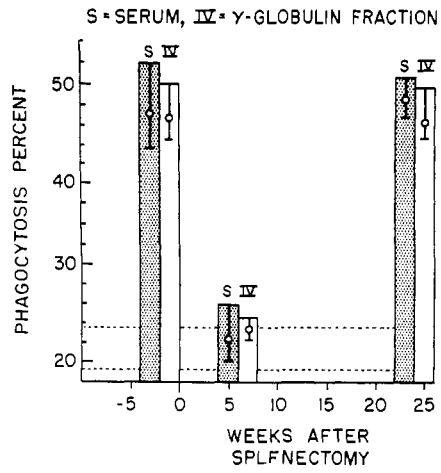

FIGURE 2: The marked loss of leucokinin activity after splenectomy. Naked polymorphonuclear leucocytes were prepared by washing the buffy coat four times with five volumes of Hank's medium at room temperature. Phagocytosis was measured in a reaction mixture composed of 0.3 ml of buffy coat, 0.05 ml of serum (S) containing 90–100 μg of fraction IV, or 0.1 mg of CP fraction IV, and 0.03–0.05 ml of an 18-hr culture of *S. aureus*. All components were dissolved or suspended in sucrose medium. Samples were taken at various times for assay. Values obtained after 30-min incubation are represented in the figure. In the absence of added serum or fraction IV the usual range of phagocytosis under these conditions was about 19–23%. This range is represented by the dotted lines shown in the graph. The values shown in the the columns represent four splenectomized untreated dogs 22, 23, 26, and 29. Not included in the graph are dogs 21 and 30 which were splenectomized and treated with 250 mg each of fractions III and IV weekly by intramuscular injection. These showed no reduction of leucokinin activity using either coated cells or naked cells with added whole serum or fraction IV. The range of phagocytosis in these three types of tests fell within 45–52%. The open circle in the column represents the average value obtained for two or more determinations on each of the four dogs represented. The bars indicate the maxima and minima of these values. Details as in the text.

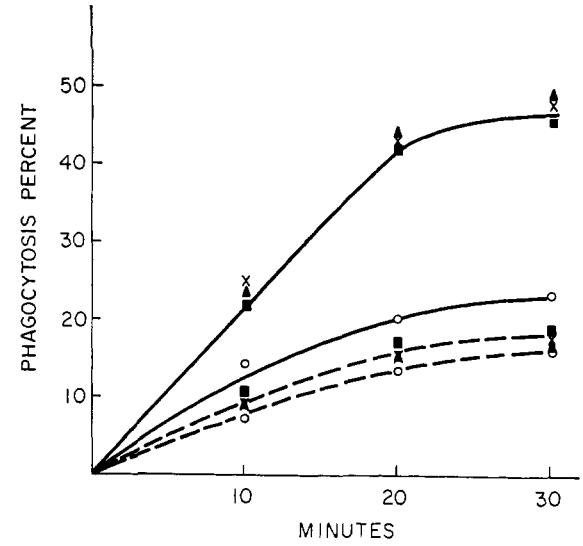

FIGURE 3: The marked reduction in the rate of phagocytosis after splenectomy. The reaction mixture was identical with that described in Figure 2. CP (0.1 mg) fraction IV prepared before removal of the spleen represented the minimum amount required for maximum stimulation under the standard conditions described. This amount was present in 0.05 ml of the corresponding serum. On that basis, the amount of serum or CP fraction IV, obtained before and 6 weeks after splenectomy, was added to the reaction mixture. Increasing the postsplenectomy samples to ten times the respective concentration, did not reveal any stimulatory effect. Coated cells in each case were isolated from the buffy coat and washed three times each with three volumes of sucrose medium at room temperature. (——) Before splenectomy. (– – – –) After splenectomy. (▲) Serum, (×) fraction IV (■) coated, and (○) naked.

phagocytic reaction was carried out in the sucrose medium which, by virtue of its low ionic strength, promotes the binding of the specific γ-globulin to the cell membrane.

Figure 2 shows the dramatic drop in the leucokinin activity obtained with serum and CP fraction IV, 4–8 weeks after splenectomy. The leucocyte, of necessity, was secured after splenectomy. By contrast, its phagocytic ability, in the presence of presplenectomy serum or CP fraction IV, without exception, paralleled that obtained before splenectomy. Included in this figure are the phagocytic activities at two periods after removal of the spleen. It is clear that spontaneous recovery was found to be complete in all animals by the 6th month after splenectomy.

The phagocytic activity of coated cells in sucrose medium before splenectomy (43–55%) was similar to that observed with naked leucocytes after addition to the latter of CP fraction IV or serum. On the other hand, coated leucocytes obtained after splenectomy gave markedly reduced values. These paralleled the low values exhibited by naked cells with added postsplenectomy serum or CP fraction IV, both of which were deficient in leucokinin activity. The range in both instances was 17–23% phagocytosis under standard conditions. The phagocytic activity of naked leucocytes, measured in the absence of added serum or CP fraction IV, was also low and showed comparable values of 18–25% before splenectomy and 15–21% after splenectomy. Figure 3 shows a kinetic study of the phagocytic activity under these conditions. Normal levels of phagocytic rates were also obtained with postsplenectomy naked leucocytes when the latter were tested in the presence of presplenectomy serum or its CP fraction IV. However, if these cells were previously coated with post splenectomy serum γ-globulin then tested in the presence of added presplenectomy serum or fraction IV, no enhancement in phagocytosis was discernible. The rate remained at about the basal level. Similarly, when prepared naked cells were subsequently coated by incubation in either presplenectomy serum or fraction IV in sucrose medium, followed by washing also with the same medium, the further addition of postsplenectomy serum or fraction IV lacking leucokinin activity, neither inhibited nor augmented the rate of phagocytosis.

The Reduction of Erythrophilic CP Fraction III and the Half-Life of the Erythrocyte. In previous experiments we observed (Fidalgo *et al.*, 1967b) that following splenectomy there was a very marked reduction of one of the γ-globulin fractions of the serum, erythrophilic CP fraction III. At the same time the half-life of the red

cell was correspondingly decreased. The change was not a permanent one and after the lapse of several months, the level of the erythrophilic fraction returned to normal with a parallel improvement in the half-life of the erythrocyte. In the process of studying the effect of splenectomy on leucokinin activity, a similar study of the levels of erythrophilic γ-globulin as it relates to the life span of the erythrocyte was undertaken. The results are presented in Figure 4 and again show the same change in both parameters running concurrently. There is a marked drop in the level of CP fraction III, 4–8 weeks after splenectomy. There is also a parallel reduction of the half-life of the erythrocyte. In this experiment, as in the previous one, there was a spontaneous recovery; both parameters becoming normal 4–6 months after removal of the spleen. The two treated dogs and the two sham-operated controls showed no reduction of either fraction III or the erythrocyte half-life.

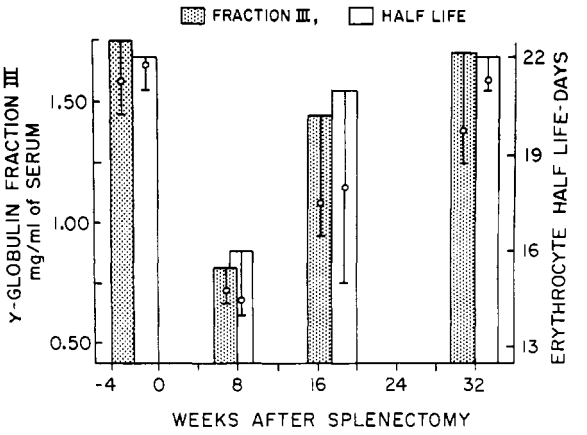

FIGURE 4: The simultaneous reduction of CP fraction III and the erythrocyte half-life after splenectomy. γ-Globulin fraction III was assayed as described in Figure 1. The half-life of the red cell was measured by ^{51}Cr tagging according to Waldman *et al.* (1960). The open circle in the column represents the averge value obtained for two or more determinations of CP fraction III on each of the four dogs represented. Only one determination each was done on the half-life at the particular intervals indicated. The bars indicate the maxima and minima of these values.

Discussion

It appears certain that splenectomy in the dog results in the virtual disappearance of the stimulatory effect of serum, or γ-globulin fraction IV prepared therefrom, on the phagocytic activity of the polymorphonuclear leucocyte. This deficiency is not a permanent one. All animals in due course and at variable intervals of time, 4–8 months after the removal of the spleen, regain their leucokinin activity to normal levels. How this is brought about is uncertain. The property of the resurgent leucokinin activity, in every manner tested, is similar to that observed before splenectomy. It is confined to CP fraction IV and is more effective in low ionic strength sucrose medium than in high ionic strength Hank's medium. It is also of the γG class (Fidalgo and Najjar, 1967b). Whether this protein is now formed in other segments of the lymphatic system or in accessory spleens cannot be ascertained at the present time. It is of special interest that after splenectomy leucophilic γ-globulin continued to be synthesized in sufficient quantity to saturate receptor sites on the leucocyte membrane. This was vividly demonstrated by the fact that once the leucocyte is coated with postsplenectomy leucophilic γ-globulin, lacking leucokinin activity, the addition of active leucokinin from presplenectomy serum fails to restore maximum activity. Maximum rates, however, can be readily attained if the leucocyte is first coated with active presplenectomy serum or CP fraction IV. It thus appears that leucophilic γ-globulin is still formed in normal amounts after splenectomy, yet is definitely lacking in leucokinin activity. It is possible that the specific leucophilic γ-globulin which is synthesized postoperatively is structurally not identical with that normally produced, even though it still preserves its specific binding characteristics.

The reduction in the half-life of the erythrocyte which appears simultaneously with the reduction of γ-globulin fraction III confirms previous findings reported from this laboratory. Both deficits also disappear in time concurrently. Most animals in this study managed to maintain normal volume and number of erythrocytes. Cell counts and hematocrits varied within normal limits, 3.9–4.5 × 10^6 per cmm and 40–45%, respectively.

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