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Research Article

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The Effect of Bacillus Calmette-Guerin-Induced Macrophage Activation on the In Vivo Clearance of Sensitized Erythrocytes

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ABSTRACT The clearance of ^{51}Cr -labeled guinea pig erythrocytes, sensitized with a known amount of IgM or IgG antibody, was examined in normal and BCG-infected guinea pigs. In normal animals, IgM-coated cells were rapidly sequestered in the liver. Most of these cells were then slowly released into the circulation where they survived normally as Coombs-positive erythrocytes. Neither the site nor extent of initial clearance showed major alterations in BCG-infected animals; however, there was no return of the sequestered erythrocytes into the circulation. This pattern of clearance was only seen in normals at very high levels of sensitization. In contrast to the IgM studies, the pattern of clearance of IgG-sensitized erythrocytes was not altered, but the rate and magnitude was markedly increased at all levels of sensitization. In addition, complement-independent clearance of IgG-sensitized erythrocytes was augmented in BCG-infected guinea pigs lacking classical complement pathway function. The spleen remained the organ primarily responsible for this increased clearance of IgG-sensitized erythrocytes. Sensitized cells in BCG-infected animals were removed from the circulation as if they were coated with several times the amount of antibody. Serum factors were shown not to be responsible for the increased clearance. These data suggest that increased macrophage activation in BCG-infected animals plays a critical role in determining the consequences of cell sensitization in vivo. These studies may help to explain exacerbations of hemolytic anemias and related states after intercurrent infections.

INTRODUCTION

In most cases the in vivo destruction of antibody-sensitized erythrocytes is not due to intravascular hemolysis,

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but follows clearance and phagocytosis by macrophages of the reticuloendothelial system (RES)¹ (1-3). The pattern and extent of clearance depends on the class of antibody coating the cells, on the ability of the antibody to activate complement, and on the presence of specific receptors on cells of the RES (3-6). If the antibody is noncomplement fixing, the presence of a specific antibody receptor is required (4-6). If the antibody binds complement, activation of the complement cascade leads to deposition of the complement fragment C3b on the erythrocyte membrane surface (7, 8). Fixed macrophages of the RES have receptors for C3b, and the IgG Fc fragment and erythrocytes with C3b or IgG on their surface adhere to these phagocytic cells (9-13).

We have previously described an experimental model of immune hemolytic anemia in the guinea pig, designed to evaluate in molecular terms the separate functions of antibody, complement, and the RES in the clearance and destruction of sensitized erythrocytes (5, 6). Studies using this model have demonstrated distinct clearance patterns for erythrocytes coated with IgG and IgM antibodies (5). Clearance of IgM-coated erythrocytes was absolutely complement dependent, while clearance of IgG-coated cells was increased many fold in the presence of complement activation (4-6, 14). This experimental model also permitted quantitative assessment of the effects of various therapeutic modalities on the destruction of the antibody-sensitized erythrocytes. Thus, corticosteroid therapy reduced clearance of IgG- and IgM-sensitized cells, while splenectomy only influenced the clearance of IgG-sensitized cells (15). In these cases the distinctive pattern of clearance remained unchanged with therapy, but sensitized erythrocytes were cleared as if they had fewer antibody molecules per cell.

¹Abbreviations used in this paper: C4D, C4-deficient; CFA, complete Freund's adjuvant; RES, reticuloendothelial system; $t_1/2$, mean half time of survival.

One action of corticosteroids is to decrease the metabolic activity and function of macrophages (15, 16). It was of interest, therefore, to determine whether stimulation of the RES would alter erythrocyte clearance.

It is well known that exacerbations of hemolytic anemia may accompany an infectious illness (1, 3). Infections alter many physiologic parameters, but recently, certain intracellular infections have been shown to influence profoundly the state of macrophage activation (17). Macrophages so altered have been shown to have increased metabolic, phagocytic, and bactericidal activity (17-23). Moreover, activation of macrophages is associated with increased ability of the RES to clear inert particles (22, 23). In this report, we document the effects of systemic BCG infection in the guinea pig on the clearance of antibody-coated erythrocytes. Striking alterations in the clearance of IgG- and IgM-sensitized erythrocytes are demonstrated, and the changes induced by macrophage activation are shown to be distinctive for each of the immunoglobulin classes.

METHODS

The preparation and source of buffers, complement reagents, guinea pig erythrocytes, and rabbit antiginea pig erythrocyte antisera have been previously described, as have the methods of immunoglobulin purification and complement studies (5, 6, 24-26).

Animals. All studies utilized either outbred 300-500 g guinea pigs from a colony known as "NIH multipurpose" or a subline of these animals with a genetically controlled total deficiency of the fourth component of complement (14).

Bacillus Calmette-Guerin. The BCG was the Phipps strain, Trudeau Mycobacterial Collection, no. 1029. After rapid warming from -70° to 37°C , the organisms were diluted to a concentration of 6,000,000 colony-forming u/ml with sterile saline. 1 ml of this solution was injected intravenously via the hind foot vein, while control animals received 1 ml of sterile saline.

Complete Freund's adjuvant (CFA). 0.1 ml of CFA (Difco Laboratories, Detroit, Mich.), emulsified with an equal volume of 0.85% saline, was injected subcutaneously in both fore and one hind foot pad and in the nape area.

Clearance studies. Studies were performed 8-14 days after BCG infection, except in kinetics experiments designed to compare alteration in clearance at various times post-infection. During this period, the initial rate and extent of clearance remained stable. The technique employed in these studies has been previously described in detail (5, 6), and only a brief outline will be included here. Normal guinea pig erythrocytes labeled with ^{51}Cr were sensitized with highly purified high avidity rabbit IgG or IgM antibodies to produce a known number of complement-fixing sites as determined by the C1a fixation and transfer test. After injection of 1 cc of 2.7×10^8 sensitized or control unsensitized cells into the hind foot vein, serial 0.1-ml bleedings from the retro-orbital sinus were obtained to determine erythrocyte survival. The 0.1-ml samples were suspended in 1 ml of EDTA buffer and then counted with a gamma scintillation counter. Infected and control groups were always studied simultaneously. Groups of

animals were sacrificed 2 and 24 h after injection, and organ localization of sensitized erythrocytes was determined by counting the radioactivity of the whole organ. The clearance data were analyzed and plotted as previously described.

Effect of BCG infection and CFA administration on hematological parameters, serum complement, weight gain, and spleen and liver weights. Groups of guinea pigs were weighed every other day for 14 days after administration of BCG, CFA, or sterile saline. The animals were then sacrificed under ether anesthesia by removal of 10-15 cc of heart blood via a direct cardiac puncture with a 19-gauge small vein infusion set (Abbott Laboratories, North Chicago, Ill.). A 5-ml portion was placed in EDTA solution and the remaining blood allowed to clot for 2 h at 22°C . The serum was removed and frozen in multiple portions at -70°C for complement studies. Serum complement was measured by standard methods (24). The liver, spleen, and kidneys were removed and weighed. The erythrocyte, leukocyte, and platelet counts were performed with an electronic particle counter, Model S (Coulter Electronics, Inc., Industrial Div., Hialeah, Fla.).

RESULTS

Unsensitized erythrocyte survival in normal and BCG-infected guinea pigs. After injection of ^{51}Cr -labeled unsensitized erythrocytes into 20 normal guinea pigs, the mean half time of survival ($t_{1/2}$) was 7.5 ± 1.0 (mean \pm SE) days. An identical survival study was performed in BCG-infected animals beginning on the 7th day of infection. The $t_{1/2}$ for four normal and four BCG-infected animals studied simultaneously was 7.3 ± 0.8 and 5.6 ± 0.6 , respectively; this difference was not statistically significant ($P > 0.05$). This slightly decreased survival rate of unsensitized erythrocytes in BCG-infected animals did fall outside the 95% confidence limits as calculated by a linear regression equation for 20 normal guinea pigs between day 3 and 7 in a 14-day study.

Effect of BCG infection on the clearance of IgM-sensitized erythrocytes. In normal animals at 117 IgM C1-fixing sites per erythrocyte, between 50 and 70% of the cells are removed from the circulation by the liver within 10 min after injection. Over the next 2 h, most of these sequestered cells return to the circulation where they survive normally.

Fig. 1 is a representative study comparing the clearance pattern at 117 IgM C1-fixing sites in normal and BCG-infected animals. The latter did not sequester a significantly greater percentage of the cells; however, there was no release of the erythrocytes once trapped by hepatic macrophages. This lack of release after initial sequestration was seen in five separate studies involving BCG-infected animals at 117 IgM C1-fixing sites and in two studies at 60 sites per cell. The pattern was never observed in normals, unless the level of erythrocyte sensitization was increased to 351 IgM C1-fixing sites per erythrocyte. A clearance curve at this high level of sensitization is also included as an in-

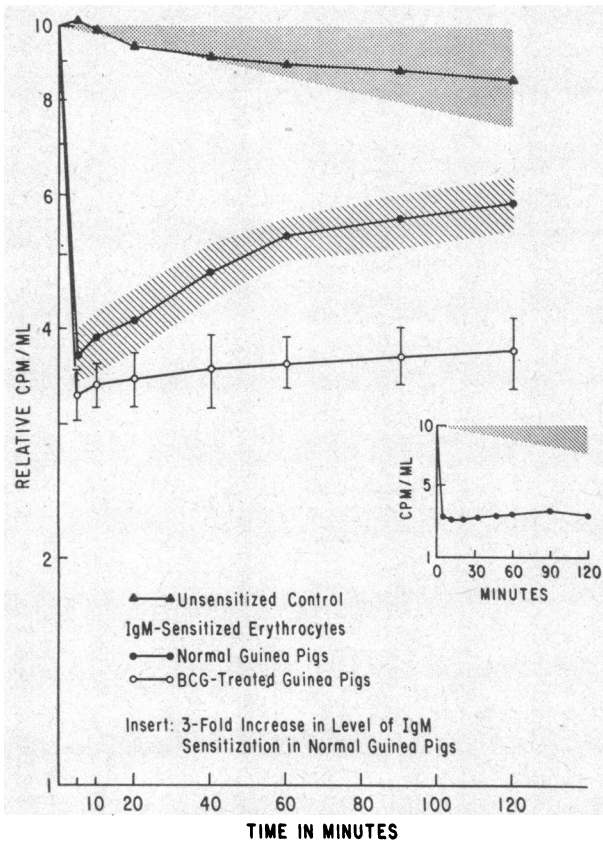


FIGURE 1 Survival of ^{51}Cr -labeled guinea pig erythrocytes sensitized with 117 IgM C1-fixing sites per cell: comparison between the mean \pm SE of four normal and four BCG-infected animals. The insert is the mean clearance curve of four normal animals at 351 IgM C1-fixing sites per cell. The shaded area in this and subsequent figures represents the 95% confidence limits for decay curve slopes of unsensitized erythrocytes in 20 normal animals.

sert in Fig. 1. The degree of initial clearance was not significantly different between normal and BCG-infected animals at 60 or 117 IgM C1-fixing sites per cell, although the mean was greater for the BCG-infected animals in each case. Erythrocytes sensitized with 117 or 234 IgM C1-fixing sites survived normally in BCG-infected, as well as uninfected, C4-deficient (C4D) guinea pigs.

Because of the lack of erythrocyte release in BCG-infected guinea pigs, there were significantly fewer cells surviving in the circulation at 2 or 24 h in this group compared to normals (Table I). Counts of tissue obtained at autopsy demonstrated the liver to contain an increased percentage of erythrocytes which was proportional to the decreased number of cells in the circulation.

Effect of BCG infection on the clearance of IgG-sensitized erythrocytes. The general clearance pattern of

IgG-coated erythrocytes is one of progressive erythrocyte trapping. With the antibody preparation used in this study, 1.0–1.6 IgG C1-fixing sites were required to detect increased clearance in normal animals. As the number of IgG complement-fixing sites was increased, a larger percentage of the cells were sequestered; at 17 IgG C1-fixing sites, greater than 90% of the cells were always removed.

Studies were performed to compare clearance curves in normal and BCG-infected animals at 0.5, 1, 2, 7, and 17 IgG C1-fixing sites per erythrocyte. In each of these cases, the pattern of clearance was typical of that seen with IgG coating of erythrocytes (Fig. 2). However, at all levels of sensitization, the rate of clearance was accelerated in BCG-infected animals. Also, at all levels of sensitization, the percent of cells surviving in the circulation at 2 and 24 h was decreased in infected guinea pigs (Table II). Sensitized cells in BCG-infected animals behaved as if they were coated with 2–3 times as much antibody and complement (Table II). Studies of organ localization at 1 and 2 IgG C1-fixing sites re-

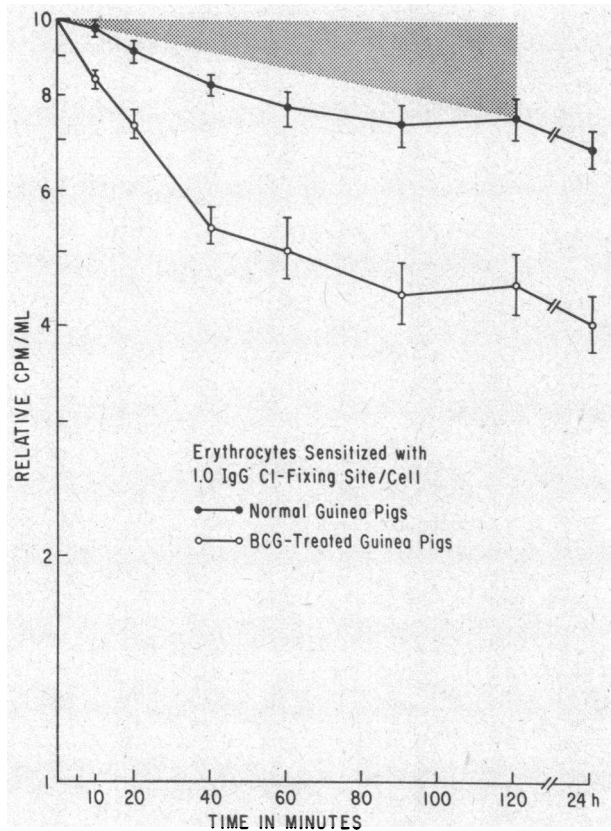


FIGURE 2 Survival of erythrocytes sensitized with 1.0 IgG C1-fixing site per cell: comparison between the mean \pm SE of five normal and five BCG-infected guinea pigs.

TABLE I
Organ Sequestration of Radiolabeled Erythrocytes 24 h After Injection*

Organ	117 IgM C1-fixing sites per erythrocyte		1 IgG C1-fixing site per erythrocyte		2 IgG C1-fixing sites per erythrocyte		17 IgG C1-fixing sites per erythrocyte	
	Normal (2)†	BCG (2)	Normal (3)	BCG (3)	Normal (4)	BCG (4)	C4D‡ (4)	BCG-C4D (4)
In circulation	60.3±3.9	42.3±3.7	74.5±3.1	45.1±4.7	56.9±7.3	12.3±5.3	56.5±6.5	16.2±5.2
Spleen	6.0±0.4	5.4±1.3	7.5±1.2	30.4±1.1	16.8±4.2	54.9±7.5	14.1±2.8	49.2±6.2
Liver	29.6±4.3	47.1±9.2	14.7±1.1	22.0±0.6	21.0±1.3	26.4±2.1	25.1±3.1	29.0±3.0
Lung	2.5±0.4	1.9±0.2	1.9±0.5	2.5±1.0	3.6±0.5	0.8±0.1	2.6±0.6	1.9±0.9
Kidney	3.1±0.4	1.8±0.3	2.6±0.2	1.8±0.2	2.5±0.3	1.5±0.3	2.6±0.4	0.8±0.2

* Percent of total counts injected.

† Number of animals in each group.

‡ Guinea pigs with a genetically controlled total deficiency of the fourth component of complement.

|| No attempt has been made to correct for radioactivity of blood within organs.

vealed a significant increase in splenic uptake in BCG-infected animals compared to normals (Table I).

To determine if serum factors were responsible for the increased clearance in infected animals, erythrocytes sensitized with 2 IgG C1-fixing sites were exposed (30 min at 37°C) to an excess of fresh serum (2 ml/ml RBC) from normal or infected animals. The clearance of these cells was then examined in normal animals. The rate and magnitude of clearance were not significantly different (percent clearance at 2 h; 46.3±4.2 BCG, and 48.4±2.2 normal).

To determine if the noncomplement-dependent clearance of IgG-sensitized erythrocytes was modified by BCG infection, comparative studies were performed in C4D guinea pigs. In these animals, at least 17 IgG C1-fixing sites per cell are required to detect decreased erythrocyte survival. Clearance at this level of sensitization was examined in uninfected C4D and BCG-infected C4D guinea pigs (Fig. 3). The percent of erythrocytes surviving at 24 h in C4D and C4D-BCG-infected animals was 58.2±4.8 and 18.4±5.3, respectively. Autopsy studies revealed increased splenic sequestration in C4D guinea pigs infected with BCG (Table I).

Effect of CFA administration on the clearance of IgG-sensitized erythrocytes. Clearance studies were performed 10 days after CFA administration, when there was marked foot pad and regional lymph node enlargement. Neither normal animals at 1 IgG C1-fixing site nor C4D's at 17 IgG C1-fixing sites had a faster rate or greater degree of clearance than saline-treated controls (Table II).

Effect of BCG infection on various hematological parameters, percent weight gain, liver and spleen weights, and serum complement (Table III). At 14 days, the significant hematological findings included a slight reduction in the hematocrit and a 40 and 44% reduction in the white blood and platelet counts, respectively. The

reticulocyte and differential leukocyte counts were similar for both groups. A more detailed analysis of the marked increase in splenic weight is below. There was also a small but significant increase in liver and kidney weights. All animals were weighed at the time of BCG injection and before being used in a clearance study. Of over 40 animals, only 3 weighed less than before the infection, and these animals were not employed in clearance studies. Most animals steadily gained weight for as long as they were followed, although at a slower rate than saline-injected litter mates. In contrast, CFA administration was unaccompanied by significant alterations in any of these parameters. Serum complement

TABLE II
Percent of Erythrocytes Surviving in the Circulation of Normal and BCG- or CFA-Treated Guinea Pigs

Number of IgG C1-fixing sites per erythrocyte	H after injection	Percent of cells surviving in the circulation		
		Normal*	BCG*	CFA*
0.5	2	87.0±6.2	78.8±5.1	
	24	83.4±5.7	61.3±4.9	
1.0	2	75.9±4.5	46.4±4.3	80.6±3.8
	24	68.2±3.8	39.2±2.9	78.6±5.9
2.0	2	56.0±4.5	17.4±4.7	
	24	51.6±4.9	7.1±2.2	
7.0	2	23.6±5.1	5.5±2.4	
	24	18.3±3.5	5.8±2.2	
17	2	6.1±1.9	4.6±2.4	
	24	3.8±1.8	1.8±0.9	
		C4D‡	C4D-BCG	C4D-CFA
17	2	74.3±5.1	24.0±7.9	72.0±2.9
	24	58.2±4.8	18.4±5.3	65.1±7.6

* This data represents the mean ±SE of at least three animals in each group. The clearance studies were performed 8-14 days after BCG or CFA administration.

‡ Guinea pigs with a genetically controlled total deficiency of the fourth component of complement.

TABLE III
*Effect of BCG Infection on Hematological Parameters, Serum Complement,
 Spleen and Liver Weights, and Percent Weight Gain*

	Normals (8)*	BCG-infected (8)
Hematocrit, ml/100 ml	37.6±0.9‡	34.2±0.5
Hemoglobin, g/100 ml	12.8±0.3	11.6±0.2
Reticulocyte count, %	2.2±0.12	1.9±0.4
White blood count§	7.0±0.8	4.2±0.4
Platelet count§	547,750±78,000	243,000±41,000
Serum complement (CH ₅₀), hemolytic U/ml	Day 0, 158±15	Day 14, 226±14
	Normals (24)	BCG-infected (24)
Liver size, g/100 g body wt	5.18±0.12	6.0±0.12
Spleen size, mg/100 g body wt	182.7±9.2	919.4±46.2
Weight gain %	26.1±1.6	18.1±1.5

* Number in each group. The data on all parameters was obtained 14 days after the onset of infection, except for hepatic and splenic weights which were obtained between 8 and 14 days after infection.

‡ Mean±SE.

§ Thousand/mm³.

|| (Final wt at day 14 - initial wt)/initial wt.

(CH₅₀) rose during the course of BCG infection. The complement titer rose from a mean of 158±15 before infection to 226±14, 14 days after infection. C3 inactivator, as measured by inhibition of immune adherence, was present in high titer in serum of BCG-treated animals.

Kinetic studies of the effect of BCG infection on clearance and on spleen weight. Utilizing 2 IgG C1-fixing sites per cell as the level of sensitization, clearance studies were performed at various times after BCG infection. Injections of BCG were staggered so that the time intervals could be studied simultaneously. As shown in Fig. 4, increased clearance of sensitized cells was evident as early as 3 days after infection. Clearance rapidly reached a maximum and did not change significantly from day 8 to 14. Also shown in this figure is the increase in spleen weight during this same period. Although this parameter generally correlated with the increase in clearance, there was significant variation early and late. At 3 days after infection, the increased clearance measured 60% of the maximal amount obtained, whereas only a minimal increase in spleen size was noted. Also, at 23-25 days the spleen size was still maximal, while there was decreased clearance compared to the 8-14-day group.

DISCUSSION

The clearance of both IgG- and IgM-sensitized erythrocytes was altered by systemic BCG infection. In the case of IgM there was a striking change in the clearance pattern; there was virtually no return of the sequestered cells into the circulation. This is in sharp contrast to the

situation in normals at equivalent levels of sensitization where most IgM-coated cells trapped by the liver are returned to the circulation where they survive normally as Coombs-positive cells.

The clearance of IgG-sensitized erythrocytes was markedly enhanced in BCG-infected guinea pigs. Not only was the rate of clearance increased, but also the percent of cells removed from circulation was increased at all levels of sensitization. Furthermore, there was augmented clearance of cells sensitized with 0.5 and 1.0 IgG C1-fixing sites per cell. The former level of sensitization is not associated with decreased survival in normals, while the latter induces only barely detectable clearance (Fig. 2). These findings suggest that augmentation in the rates of clearance of IgG-sensitized cells occurs by complement-independent as well as by complement-dependent mechanisms. This hypothesis was confirmed by the finding of increased clearance in BCG-infected C4D guinea pigs as compared to uninfected C4D animals. Antierythrocyte antibodies do not efficiently activate the alternate complement pathway, and clearance in these animals is therefore complement independent (5, 27). Experiments were performed to determine whether BCG infection might change rates of clearance by alteration of the levels of serum factors. Although complement (CH₅₀) rose after BCG infection, exposure of sensitized cells to serum from BCG-treated animals did not alter the rate of clearance. Thus, BCG appeared to influence clearance via cellular factors.

There is data to suggest that when the RES is stimulated by an increased work load (as in hemolytic anemia), estrogens, endotoxins, particulate matter (zymosan),

and intracellular infections, a series of interrelated events occur (16, 19-21, 28-30). There is a marked proliferation of macrophages which have increased metabolic activity, and the organs containing these cell types increase rapidly in size with a proportional increase in blood flow. Consequently, organs sequestering sensitized erythrocytes are larger and contain a proportionately and absolutely greater number of macrophages which are in turn activated. In comparison to unactivated macrophages, these activated cells are known to ingest more avidly foreign particles, spread over glass surfaces, and have a larger number of IgG cell surface receptors (20, 31). In the BCG-infected animals used in these experiments, increased metabolic activity of the RES was evident, in that cleared cells were catabolized with ^{51}Cr release twice as fast as in normals, as judged by the rate of ^{51}Cr release from sequestered erythrocytes.

This series of events would appear to be sufficient to explain the increased clearance of IgG-coated cells in BCG-infected animals. In the experiments reported

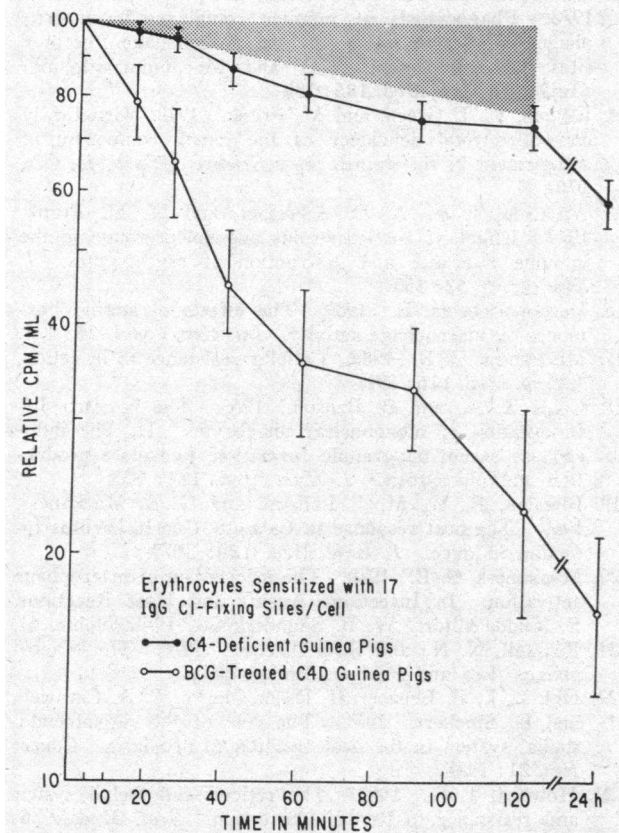


FIGURE 3 Survival of erythrocytes sensitized with 17.0 IgG CI-fixing sites per cell: comparison between the mean \pm SE of six C4D and six BCG-infected C4D guinea pigs.

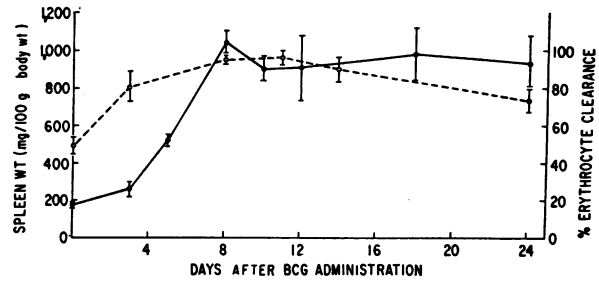


FIGURE 4 Chronological comparison after systemic BCG infection between the increase in splenic weight (\bullet) and the increase in clearance (\circ) of erythrocytes sensitized with 2.0 IgG CI-fixing sites per cell.

here, the spleen weight increased fivefold over a 1-2-wk period after infection, and a proportional increase in splenic blood flow would be expected to accompany this organ enlargement. There appear to be both rapid and delayed pathways of erythrocyte passage through the spleen, and splenomegaly has been shown to be associated with delayed splenic transit times and erythrocytosis (32, 33). Consequently, there may be more opportunity for sensitized cells with IgG and/or C3b sites to adhere to splenic macrophages and be ingested in BCG-infected animals.

In general, activation of splenic macrophages is accompanied by increased spleen size. However, two points suggest that the state of macrophage activation played an important role in addition to splenomegaly, per se. Increased rates of clearance were noted by day 3 after infection, a time at which significant splenomegaly had not yet occurred (Fig. 4). Also, as shown in earlier studies, corticosteroids decreased splenic sequestration of IgG-coated cells without altering splenic weight (15). Sensitized cells were cleared in cortisone-treated animals as if they had fewer sensitizing sites per cell. In these cases, changes in splenic size could not have been responsible for the observed effect.

Data obtained in studies of the clearance of IgM-coated erythrocytes could also be explained by this general formulation. In BCG-infected animals, IgM-coated cells were cleared as if they had far greater numbers of antibody molecules per cell; at high IgM site density, cells are cleared but not released from the liver in normal animals. Unlike IgG, no receptors for heterologous IgM have been observed on macrophages, so that macrophage adherence would be expected to require complement (9-13). The hypothesis most consistent with these data is that the number and/or function of macrophage C3b receptors is increased in BCG-infected animals. In vitro studies will be required to document these changes, but the suggestion is consistent with in vitro findings of Arend and Mannick of increased numbers

of IgG-binding sites on BCG-stimulated alveolar macrophages (31).

This study raises several points with regard to clinical hypersplenism, the role of an enlarged spleen in hemolytic anemia, and the effect of intercurrent infections in patients with hemolytic anemia. BCG-infected animals had massive splenomegaly and some features characteristic of the hypersplenic state. They had a modest decrease in normal erythrocyte survival and mild neutropenia and thrombocytopenia. However, the reticulocyte count was normal, and the hematocrit showed only a minimal decrease, suggesting that hemolytic anemia was not a major component of this syndrome. There have been a number of published reports of hemolytic anemia apparently solely due to an enlarged spleen, but these are quite unusual (34, 35). Often, careful analysis of these cases reveals the presence of an erythrocyte defect or low levels of erythrocyte sensitization (35, 36). Thus, the clinical data, as well as our own, suggest that an enlarged spleen is a more efficient filter of lightly damaged or mildly abnormal erythrocytes, but tends to spare normal erythrocytes. The clinical association of an exacerbation of hemolytic anemia, concomitantly or after an infectious illness (1, 3), may have a parallel in data presented here. If these patients develop RES activation as a consequence of an associated infection, especially with splenomegaly, there may be an exacerbation of their clinical illness. Similar reasoning may explain the exacerbation of hemolytic anemia associated with pregnancy (1, 3), since estrogens are known to activate the RES and increase the clearance of inert particles in experimental animals (16). In summary, the data illustrate how intercurrent infection may alter the fine balance which regulates the extent of immunologically mediated damage in vivo.

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