Studies of in Vivo Phagocytosis. I. Inhibition of Phagocytosis by Intact Neoplastic Body Fluids

By Sanford G. Kimball and Jerome I. Brody

The purpose of this study was to delineate additional factors related to the pathogenesis of uncontrolled infections in patients with leukemia and reticuloendothelial disorders by determining, using a laboratory prototype, whether their serums and urines inhibit in vivo rabbit leukocyte phagocytosis. This hypothesis was tested by a modification of the skin window technic in which the simultaneous morphologic and phagocytic response of rabbit leukocytes to external stimuli may be observed. In essence, inert (rice starch) and potentially toxic (Candida) particulate antigens were suspended in patient serums or, under special circumstances, patient urines, and applied to localized, rabbit ear abrasions over which sterile cover slips were placed. The cover slips were changed at specific intervals, appropriately stained and the resultant cell-antigen exudates present on the undersurface of the cover-slip examined microscopically.

Methods and Materials

A. Patient group: There were 24 patients ranging in age from 8–75 years with various hematologic diseases, and 27 hematologically normal patients included in this study. All were males except one female with Hodgkin's disease. In addition, one 8-year-old boy, also with Hodgkin's disease, was part of the series. Of the total of 24, 18 were receiving some form of antineoplastic treatment at the time the investigative procedures were performed. The disease types are summarized in table 1.

B. Antigens:

1. Rice starch. This substance is a satisfactory prototype to test phagocytic potential because it is inert, nontoxic and its phagocytosis requirements have been determined. The starch was washed five times with normal saline and made up to a 4 per cent suspension in the patient fluids described below.

2. Candida albicans. This organism has been used in these experiments because it is a potential pathogen and frequently complicates the clinical course of patients with neoplastic disease. The Candida were obtained from cultures not more than 24 hours old grown on Sabouraud's media by agitating 5 ml. normal saline in the culture tube. The Monilia in the opalescent fluid were washed five times and treated in a manner similar to that for starch.

Both antigens have the distinct advantage of being easily visible before and after phagocytosis. In addition, the experiment supplies all elements necessary for this type of phagocytosis since humoral antibody to both starch and monilia appears to be adequate in patients with these blood abnormalities and complement will be provided by serum exuding from the rabbit ear excoriation.

C. Suspending media:

1. Serums. Sufficient blood from control and leukemia patients was aspirated into a dry, sterile glass syringe so that after standing in a test tube for 2 hours at room temperature...
and 4 hours at 4 C., 5 ml. serum could be obtained by centrifugation. The serum, when not used immediately, was stored in glass vials at -35 C.

2. Urines. Aliquots of 24-hour urine collections from patients with multiple myeloma excreting Bence Jones protein and normal urines also were used as suspending media. Prior to the test the urine pH was adjusted to 7.0–7.4 to avoid introduction of another experimental variable which might influence phagocytic activity.

D. Ear abrasion: After shaving the dorsal ear surface of an albino rabbit, abrasions, 2 mm. square, were made with a sterile scalpel. The ears were scraped gently until the pin-point capillaries of the dermis were visible and a small amount of serum oozed from the area of trauma.

E. Performance of the test: One drop each of patient and control antigen suspensions from a Pasteur pipet were placed on opposite ear abrasions of a single rabbit so that all experiments were performed in parallel for comparative purposes. The 22 x 22 mm. sterile cover slips and an identical-sized cardboard square were taped firmly over the ears and changed at 2, 4, 6, 8, 24, 26, 28, 30 and 48 hours, each time with the addition of a drop of antigen suspension. The cover slips were air-dried, stained with Wright's stain and mounted on labeled slides. Preparations were evaluated carefully by scanning with the low power objective at each interval for the distribution of rabbit polymorphonuclear and mononuclear cells which had migrated on to the cover slips. Under oil immersion, the type of cell, its morphologic integrity, and an estimate (poor, minimal, moderate, marked) of the intensity of the cellular reaction were noted. In addition, the cell type performing phagocytosis and the amount of free starch or Candida not ingested also were recorded. A precise quantitation of results in terms of per cent cells counted and those showing phagocytosis (phagocytic index) could not be achieved, mainly because in vitro homogeneous cellular-antigen suspension ratios could not be duplicated in these in vivo preparations. Cellular migration on the cover slip and antigen suspensions, although sufficient for interpretation, were not evenly dispersed and adequacy or inadequacy of phagocytosis was evaluated by comparing each patient assay to its own, paired normal control preparation.

RESULTS

The preparations had the following general characteristics. The starch particles were refractile and did not stain with the dye. The Monilia generally took a basophilic stain but their staining properties became erratic after they remained on the rabbit ear for more than 24 hours. In most, but not all, instances the type of cellular exudate was easily reproduced and the time sequence for the appearance of the cells in both patient and control cover slips showed little variation. At 2 hours there was a notable lack of cellularity, but at 4 hours a moderate number of mature polymorphonuclear
leukocytes were noted, and this was especially marked at 6 and 8 hours. The 24-, 26- and 28-hour preparations showed marked and identifiable cellularity of the same type, but those at 30 and 48 hours were, for the most part, composed of degenerating mature polymorphonuclear leukocytes. In the latter instances, however, macrophages were present in moderate numbers. On some occasions, both with leukemic and control sera, macrophage infiltration on the coverslips was minimal which probably was related to the rabbit response per se and not to the addition of inhibitory materials. Both mature polymorphonuclear leukocytes and macrophages, when suspended in normal media, participated in phagocytosis to the point that the majority of the antigens were intracellular in the direct vicinity of the cells and few free antigen particles were present (figs. 1 and 2). On the other hand, with neoplastic suspending media, more particles were present outside than inside the cells and leukocytes and antigens were observed adjacent to one another without apparent active white cell phagocytosis.

Specifically, in four of six patients with Hodgkin’s disease, serum inhibition of phagocytosis was demonstrated. Two of these patients were receiving steroids and one inhibited starch and the other Monilia phagocytosis. Anti-
Fig. 2.—In this instance the cellular exudate is composed entirely of macrophages which are characterized by large, vesicular, folded nuclei with finely granular, vacuolated cytoplasm. In the center cell, the starch particle (large arrow) may be seen within the cytoplasm. In other instances (small arrow) the starch is also within the cell but appears to be superimposed on the cell nucleus. Note the absence of free, extracellular starch granules.

tuberculous, but not antileukemic, therapy was being given to one female patient with active miliary tuberculosis and Hodgkin's disease who inhibited both starch and Candida phagocytosis. Of the two other patients with Hodgkin's disease and normal phagocytosis, one was receiving steroid therapy and the other was not being treated.

The sera of all three patients with chronic lymphocytic leukemia inhibited phagocytosis. Candida phagocytosis was inhibited by one patient receiving vincaleukoblastine. The other patients, one of whom was taking prednisone and the other chlorambucil and prednisone, prevented normal phagocytosis of both Monilia and starch. Of the sera from six patients with paraproteinemias, all four with multiple myeloma reacted normally in phagocytosis although three were receiving external x-irradiation to the spine, prednisone or chlorambucil. However, the urine from the patient receiving localized x-irradiation inhibited Monilia phagocytosis as opposed to effectual antigen ingestion when normal urine was used as the suspending medium. One patient with primary macroglobulinemia demonstrated inhibition of Monilia phagocytosis during treatment with penicillin and streptomycin for pneu-
Table 2.—Correlative Summary of Experimental Results

<table>
<thead>
<tr>
<th>Disease</th>
<th>Phagocytosis inhibited</th>
<th>Therapy</th>
<th>Active Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin's disease</td>
<td>Candida starch</td>
<td>prednisone, INH, PAS, streptomycin</td>
<td>miliary tuberculosis</td>
</tr>
<tr>
<td></td>
<td>no candida, starch</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>Candida starch</td>
<td>prednisone</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>no candida, starch</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Macroglobulinemia</td>
<td>Candida starch</td>
<td>prednisone, chlorambucil</td>
<td>pneumonia, organism</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>no</td>
<td>undetermined</td>
</tr>
<tr>
<td>Multiple myeloma (serum)</td>
<td>no</td>
<td>prednisone, chlorambucil</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>x-ray to lumbar spine</td>
<td>no</td>
</tr>
<tr>
<td>Multiple myeloma (urine)</td>
<td>starch</td>
<td>prednisone, chlorambucil</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>no candida, starch</td>
<td>x-ray</td>
<td>no</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>no</td>
<td>prednisone</td>
<td>no</td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>Candida starch</td>
<td>6-MP, prednisone</td>
<td>no</td>
</tr>
<tr>
<td>Malignant lymphoma</td>
<td>Candida starch</td>
<td>prednisone</td>
<td>no</td>
</tr>
<tr>
<td>Reticulum cell sarcoma</td>
<td>no</td>
<td>prednisone</td>
<td>no</td>
</tr>
<tr>
<td>Chronic granulocytic leukemia</td>
<td>no</td>
<td>digoxin</td>
<td>no</td>
</tr>
<tr>
<td>Myeloid metaplasia</td>
<td>no</td>
<td>none</td>
<td>no</td>
</tr>
</tbody>
</table>

monia, and the other, taking prednisone, did not show this alteration. The acute stem cell leukemia serum inhibited phagocytosis of both starch and Monilia at the time the patient was receiving prednisone and 6-mercaptopurine. One patient with an undifferentiated malignant lymphoma, also receiving prednisone, inhibited phagocytosis of both antigens.

In contrast, sera from the patients with aplastic anemia and reticulum cell sarcoma did not reveal phagocytic inhibition although all the patients were being treated with prednisone. Sera from all the patients with chronic granulocytic leukemia and myeloid metaplasia who were receiving no specific therapy did not inhibit phagocytosis. These results are summarized in table 2. It should be stated that cellular migration, as such, was never inhibited by any antigen suspension. Moreover, discordant inhibition of the early (polymorphonuclear) and late (macrophage) components of phagocytosis did not occur.

Discussion

The most important observation of this study was that particulate antigens, sensitized by certain neoplastic serums and urines, were not ingested by rabbit leukocytes, suggesting that coating of the antigen in this fashion
impairs physiologic phagocytosis. The suppression of normal phagocytosis in these experimental procedures may be due to several endogenous and exogenous factors which act separately or in combination to interfere with the leukocyte membrane alterations suggested as being fundamental to this process.\textsuperscript{8,10} It is conceivable that the unidentified substance or substances may take several forms, one of which may be a paraprotein. The abnormal phagocytic response to antigens coated with one of the macroglobulinemic serums and the recent description of similar in vitro results with hypergamaglobulinemic myeloma plasmas supports, but does not necessarily prove, this concept.\textsuperscript{11} The failure of the four multiple myeloma serums in this study to restrain phagocytosis may indicate a difference of in vitro and in vivo leukocyte activity or that only certain, and not all, paraproteins are inhibitory. The observation that the urine from two patients excreting Bence Jones protein did alter phagocytosis is in accord with the idea that the serum and urinary proteins in multiple myeloma may be related but are not identical.\textsuperscript{12} It is of considerable interest, however, that normal urine supported unrestricted phagocytosis without the apparent availability of conventional humoral antibody. The theory of other leukotoxic or leuko-inhibitory substances may be extended by in vitro experiments demonstrating that circulating leukemic polymorphonuclear leukocytes possess less phagocytic activity than normal cells\textsuperscript{13} and that normal leukocyte phagocytic activity and morphology are altered when these cells are suspended in plasmas from patients with acute and chronic granulocytic leukemia.\textsuperscript{14}

It has been implied that the various chemotherapeutic agents used to treat malignant blood disorders suppress body defense mechanisms and may be a factor in the dissemination of infection. The observation that phagocytosis was inhibited by sera from patients receiving adrenal steroids, oral nitrogen mustards and antimetabolites is consistent with this hypothesis. Adrenal corticosteroids may depress in vivo function of the reticuloendothelial system\textsuperscript{15} and also decrease the in vitro phagocytio activity of the human polymorphonuclear leukocyte.\textsuperscript{16} Furthermore, in high doses, triethylene thiophosphoramide inhibits the in vitro migration of leukemic leukocytes.\textsuperscript{17} The inhibitory influence of serum from patients receiving antituberculous drugs and penicillin may be evidence of a similar or related toxic mechanism and may be relevant to the associated role of the broad spectrum antibiotics in disseminated mycoses.\textsuperscript{18}

There are several unexplained, correspondent observations which indicate the multifaceted nature of the problem of deficient immunologic response in these patients.\textsuperscript{19-21} In the first place, serums which inhibited \textit{Candida} phagocytosis did not necessarily prevent the ingestion of starch, suggesting that under certain conditions the living microorganism itself, perhaps by the secretion or synthesis of an exotoxin or endotoxin,\textsuperscript{22} is toxic to the cells of the induced exudate. On the other hand, the reverse—inhibition of starch and not \textit{Candida} phagocytosis—also was noted. Furthermore, despite the fact that both patients with reticulum cell sarcoma and the one with aplastic anemia were receiving prednisone, none of their serums inhibited particulate
phagocytosis. Moreover, none of the four serums from the patients with myeloproliferative disorders had a deleterious effect on cellular phagocytic activity. In addition, it is pertinent that only two patients whose serums inhibited normal phagocytosis had active infection at the time of the experiments, suggesting that the phagocytic inhibitory factors probably are operative prior to the onset of infection rather than occur as the result of this process. Finally, it is apparent that, since the intact body fluids actually are a composite of substances, the performance of similar procedures in which the suspending media would be individual components of the patient serums and urines might facilitate further detailing of the factors operative in this type of inhibition.

The observation that there is interference with the ingestion phase of particulate phagocytosis by rabbit leukocytes under experimental conditions does not necessarily mean that such events pertain to human white cells. It is suggested by analogy, however, that internal (serum) and external (chemical) factors with varying degrees of potency, acting in various combinations, may modify similarly the human phagocytic response and predispose these patients to widespread, therapy-resistant mycotic or bacterial infections.

**Summary**

1. By means of a modified skin window technic, the phagocytic response of rabbit leukocytes to external stimuli has been observed.

2. Suspensions of particulate antigens, such as rice starch and Candida albicans, made in serums or urines from patients with leukemias and lymphoproliferative disorders, were not phagocytized normally when compared to similar suspensions in normal media.

3. These results suggest that exogenous or endogenous factors, individually or in combination, interfere with normal phagocytic activity in the rabbit and, by analogy, may exercise a similar function in the human.

**Summario in Interlingua**

1. Per medio de un modificate technica a fenestra cutanea, le responsa phagocytic de leucocytos de conilio a stimulos externe esseva observate.

2. Suspensiones de antigenos particulate—amylo de ris e Candida albicans—facite in seros o urinas ab patientes con leucemias e disordines lymphoproliferative, non esseva phagocytisate normalmente in comparation con simile suspensiones in medios normal.

3. Iste resultatos suggere que factores exogene o endogene—individualmente o in combination—interfere in le normal activitate phagocytic in le conilio e—per analogia—exerce possibilemente un simile function in le homine.

**REFERENCES**


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