Failure of Pretreatment with Glucocorticoids to Modify the Phagocytic and Bactericidal Capacity of Human Leukocytes for Encapsulated Type I Pneumococcus

FRED ALLISON, JR., AND MARTHA H. ADCOCK

*Division of Infectious Diseases, Departments of Medicine and Microbiology,
University Medical Center, Jackson, Mississippi*

ABSTRACT

ALLISON, FRED, JR. (University Medical Center, Jackson, Miss.), AND MARTHA H. ADCOCK. Failure of pretreatment with glucocorticoids to modify the phagocytic and bactericidal capacity of human leukocytes for encapsulated type I pneumococcus. J. Bacteriol. 89:1256–1261. 1965.—The influence of glucocorticoid administration for 4 days upon the bactericidal and phagocytic functions of leukocytes recovered from venous blood of healthy human beings was evaluated within an in vitro system that contained fully virulent, type I pneumococcus. Ingestion of pneumococcus by granulocytes spread upon a suitable surface, followed by thin slide cultures of the preparation, permitted precise quantitation of cellular phagocytic functions. It was found that granulocytes from healthy donors subjected to short-term glucocorticoid treatment ingested and killed pneumococcus as well as did cells obtained before therapy. Human leukocytes obtained after treatment with prednisolone functioned in a suspending medium of high ionic strength as well as did cells harvested before treatment, although the efficiency of both groups was significantly less than when manipulated in autologous serum.

Enhancement of bacterial infections resulting from therapy with glucocorticoids has been thought to result from their general suppressive effect upon inflammation and the general response to injury (Thomas, 1953). The most notable of these effects can be seen in the curtailment of exudation; in particular, numerous investigators have recorded suppression of leukocytic mobilization (Allison, Smith, and Wood, 1955; Boggs et al., 1964). Most evidence of this sort has come from experiments with laboratory animals subjected to massive amounts of steroids far in excess of therapeutic levels used in man. In this regard, it should be emphasized that in many clinical examples in which bacterial infections have complicated steroid usage, the daily maintenance has not been exceptionally high. More often, secondary bacterial diseases have emerged after a program of moderate steroid dosage maintained for an extended time, and, under these circumstances, the vigor of leukocytic exudation with pus formation has not been impaired. Even so, no convincing evidence has been presented to show that treatment of human beings with glucocorticoids interferes with either ingestion or destruction of microorganisms by phagocytes.

Perhaps pertinent to this point, Hirsch and Church (1961) found that pretreatment of rabbits with cortisone acetate for as long as 10 days failed to alter the killing efficiency of polymorphonuclear leukocytes in vitro for either staphylococcus or certain salmonella. The need for clarification with reference to clinical problems made it urgent to examine this reaction in man.

The following experiments were performed in vitro with leukocytes obtained from peripheral venous blood of healthy human donors subjected to several schedules of pretreatment with prednisolone. It was found that pretreatment with glucocorticoid for 4 days failed to change the phagocytic and killing capability of human cells.

MATERIALS AND METHODS

Healthy donors. Ten male medical students between 22 and 28 years of age served as professional donors. Before being accepted for experimentation, each subject was carefully questioned for evidence of active disease and examined as indicated from the history.

Individuals with histories suggestive of recent illness or with a past record of chronic infection such as tuberculosis were not used. Contraction of an infectious disease eliminated donors from a

given phase of an experiment. A period of convalescence for at least 6 weeks was observed before donors were employed again, and, where indicated, a more extended moratorium was effected. Since each subject served as his own control, reuse was desirable in order to complete each series of experiments.

The following laboratory procedures were used routinely for screening. Skin tests were performed intracutaneously for tuberculosis with purified protein derivative (PPD) through the second-strength concentration (0.005 mg) whenever necessary to determine the full extent of skin reactivity. Students with strongly positive reactions to PPD or a documented history of conversion from negative to positive reaction within the preceding 12 months were eliminated from consideration. Roentgenograms of the chest were obtained immediately before experimentation was begun and 6 weeks after each course of steroid administration. The hematocrit, total peripheral white blood cells, and differential white blood cell count were performed on venous blood initially and repeatedly on each subject throughout the time of study. Deviation of counts from normal ranges at the initiation of an experiment led to termination of that phase of the study. Total venous blood eosinophil counts were performed according to the method of Pilot (1950) after a course of steroids to make certain maximal drug action had been achieved.

Glassware, solutions, and equipment. Unless stated otherwise, details of techniques employed have been described previously and will not be recounted here unless of particular relevance to recorded observations. As described elsewhere, the buffer of high ionic strength shall be referred to as the "original" phosphate buffer (Allison and Adcock, 1964).

Bacterial cultures. The same fully virulent strain of type I pneumococcus was propagated and otherwise handled as described previously save for the steps in washing (Allison and Adcock, 1964). The centrifugate of a 4-hr culture was resuspended in 2.0 ml of autologous serum and then centrifuged again at 3,000 rev/min or 1,500 $\times$ g for 30 min at 4 C. After supernatant serum was discarded, a bacterial count was performed (Allison and Adcock, 1964) to determine the volume of culture required to give 2×10^{10} organisms per milliliter. Viability of the final bacterial preparation was assayed in slide cultures (Allison and Adcock, 1964) and was always greater than 90%.

Preparation of leukocytes. The same procedure was followed for all groups of donors. Sterile venous blood obtained the day preceding experimentation was allowed to clot and retract in sterile containers; supernatant serum was removed for storage at 4 C.

On the day of an experiment, blood from an antecubital vein was drawn into sterile, siliconed syringes that contained sufficient ethylenediaminetetraacetate to give a final concentration of 0.12% for anticoagulation. Before blood was dispensed into siliconized conical glass tubes, a small volume of 6% clinical dextran (Abbott Laboratories, North Chicago, Ill.) was added according to the method of Hirsch and Church (1960). Sedimentation of erythrocytes at 25 C for 2 hr yielded a supernatant plasma rich in leukocytes and platelets. The plasma was removed and centrifuged at 800 rev/min for 5 min. Most platelets remained in the supernatant plasma and could be decanted easily. The button of white blood cells was then cautiously dispersed in 2.0 ml of autologous serum, enumerated in a Spencer bright line counting chamber, then centrifuged as before. The final button of cells was then ready for mixing with bacteria. Differential counts from smears of this preparation revealed about 70% polymorphonuclear granulocytes (PMNG).

Assays of leukocytic viability with trypan blue supravital stains indicated that better than 90% of cells were alive after their separation. This was further supported by the bactericidal efficiency after ingestion of type I pneumococcus. Survival of leukocytes after incorporation into agar slide cultures was not measured.

Conditions of phagocytosis and bactericidal assay. Bacteria and phagocytes were mixed in a 2:1 ratio and spread on a filter paper surface for 30 min to permit ingestion of bacteria. The method for estimating phagocytosis has been recorded (Allison and Adcock, 1964). A modification of the method of Smith and Wood (1947) was used to assess the killing of ingested bacteria (Allison and Adcock, 1964).

Analysis of statistical results was according to the method of Croxton (1953) for determining the significance between two sample means. Since it was not possible to perform an unlimited number of experiments with each subject, results from a group of studies were combined and subjected to statistical treatment.

RESULTS

Influence of short-term treatment with glucocorticoids upon the phagocytic and bactericidal capacity of leukocytes from healthy human donors. In the beginning, it was necessary to determine the phagocytic and bactericidal potential of PMNG recovered from venous blood of human donors free from disease both before and after a short program of treatment with a glucocorticoid. In the first series of experiments summarized in Table 1, pretreatment values were established, and the donor then was given 10.0 mg of prednisolone by mouth every 6 hr; the treated serum specimen was obtained after 3 days of treatment, and leukocytes were harvested on the 4th day of therapy. Under the conditions of this system it may be seen (Table 1) that administration of prednisolone failed to alter the vigor of surface phagocytosis when the cells were suspended in autologous serum obtained either before or during therapy.

Bactericidal activity by PMNG from donors

TABLE 1. *Influence of 4 days of pretreatment with oral prednisolone, 40 mg daily, upon the phagocytic and killing activity of PMNG obtained from venous blood of healthy human donors**

Donor subject number	Intensity of phagocytosis by PMNG			Bactericidal capacity of PMNG		
	Pretreatment cells and pretreatment serum (A)	Treated cells and treated serum (B)	Treated cells and pretreatment serum (C)	Pretreatment cells and pretreatment serum (D)	Treated cells and treated serum (E)	Treated cells and pretreatment serum (F)
	%	%	%	%	%	%
2	89	72	—	86	97	—
3	80	58	30	92	91	100
4	62	65	65	91	63	81
5	71	14	18	95	93	100
6	37	45	48	94	91	98
9	48	43	54	79	96	97
10	40	33	44	97	73	96
Mean values with SD	61 ± 18.6	47 ± 18.1	43 ± 15.4	91 ± 5.4	86 ± 12.0	95 ± 6.6

* The statistical significance of differences between mean values for the various groups has been expressed as follows: (1) A − B = <0.25 but >0.2; (2) A − C = <0.1 but >0.05; (3) B − E = <0.8 but >0.7; (4) D − E = <0.3 but > 0.25; (5) D − F = <0.25 but >0.2; and (6) E − F = <0.2 but >0.1.

TABLE 2. *Influence of certain electrolyte solutions and pretreatment with prednisolone upon the phagocytic and killing capacity of PMNG from venous blood of healthy human donors**

Donor subject number	Sorensen's buffer as suspending medium		"Original" phosphate buffer as suspending medium					
			No prednisolone treatment		20.0 mg of prednisolone daily for 3 days		40.0 mg of prednisolone daily for 3 days	
	Phagocytosis by PMNG (G)	Bactericidal activity of PMNG (H)	Phagocytosis by PMNG (I)	Bactericidal activity of PMNG (J)	Phagocytosis by PMNG (K)	Bactericidal activity of PMNG (L)	Phagocytosis by PMNG (M)	Bactericidal activity of PMNG (N)
	%	%	%	%	%	%	%	%
11	44	94	28	71	—	—	—	—
12	34	98	—	—	—	—	—	—
13	50	91	31	68	—	—	—	—
14	43	88	15	71	7	68	20	66
15	—	—	7	65	14	69	—	—
16	—	—	32	64	—	—	14	62
17	—	—	—	—	17	70	—	—
Mean value with SD	43 ± 5.7	93 ± 3.7	23 ± 9.9	68 ± 3.3	13 ± 3.6	69 ± 0.82	17 ± 3	64 ± 3.0

* Statistical significance of differences between mean values for the various groups has been expressed as follows: (1) G − I = <0.02 but >0.01; (2) H − J = <0.1 but >0.05.

treated with 40.0 mg of prednisolone daily for 4 days was not significantly curtailed (Table 1). It must be concluded, therefore, that short-term glucocorticoid therapy failed to compromise the phagocytic and killing capacity of PMNG from healthy human donors for encapsulated, mouse-virulent, type I pneumococcus.

Influence of short-term prednisolone therapy and certain electrolyte solutions upon the phagocytic and bactericidal capacity of leukocytes from healthy

human donors. In view of the failure to find a consistent effect of glucocorticoid pretreatment on the phagocytic and killing capacity of PMNG obtained from venous blood of healthy human donors, it was of interest to determine whether these cells would be influenced by exposure to electrolyte solutions of different concentrations. In contrast to studies described in the preceding section, PMNG harvested from freshly shed, healthy, human venous blood were washed and

suspended in either Sorensen's phosphate buffer or in the "original" phosphate buffer of high ionic strength (Allison and Adcock, 1964). The viability of extracellular pneumococcus exceeded 90% in all experiments and therefore has not been included in Table 2. It is readily apparent (Table 2) that the phosphate buffer of high ionic strength impaired both phagocytosis (P = <0.02 but >0.01) and killing (P = <0.1 but >0.05) of engulfed organisms to a significant degree.

Pretreatment of donors with prednisolone, however, at levels of either 5.0 or 10.0 mg every 6 hr for 3 days failed to enhance the defect produced by the "original" phosphate buffer of high ionic strength. Since the pattern of the data was consistent and failed to reveal a difference of major proportions from that of the untreated PMNG, a large number of samples was not obtained. Statistical treatment of these values was not performed.

DISCUSSION

Careful examination of the phagocytic and bactericidal capacity of blood-borne leukocytes obtained from both healthy human donors treated with prednisolone failed to reveal consistent impairment of either function. This was so regardless of the type of medium employed for suspending harvested cells. The most detailed studies were performed with PMNG handled exclusively in autologous serum in the hope that both viability and function of cells would be preserved to a maximal degree. In order to preserve opsonizing and other humoral antibacterial substances under these circumstances, autologous serum was not heated, thus assuring test conditions as nearly like those that prevail in vivo as possible.

When cells from healthy human beings treated with glucocorticoid were handled in a buffer of near physiological ionic strength, such as Sorensen's phosphate solution, both phagocytic and bactericidal functions were maintained at high levels. On the other hand, after exposure of PMNG from either untreated or treated donors to an unphysiological solution, in this instance the high ionic "original" phosphate buffer, both groups of leukocytes were inhibited substantially but to an equal degree. This was in contrast to the effect of the high ionic "original" buffer upon PMNG recovered from peritoneal exudate of rats treated with hydrocortisone (Allison and Adcock, 1964), for these cells were distinctly less efficient than were white blood cells secured from untreated animals. An explanation for differences in response to the same conditions between species is not readily apparent; however, it serves to emphasize hazards inherent in the extrapolation of experimental results from laboratory animals to man.

Before concluding categorically that neither phagocytosis nor bacterial killing by PMNG derived from venous blood is altered by administering glucocorticoids to human beings, several reservations that apply to our results should be observed. In the first place, it must be pointed out that protracted glucocorticoid therapy of moderate dosage administered to healthy human donors would have approximated more closely the clinical circumstances usually extant when complicating infections are encountered. It has not been possible for us to undertake such a study since a suitable group of donors has not been conveniently available. Patient donors have not proved a satisfactory substitute in this regard, because of the nature of their primary disease. In each of a series of subjects studied but not reported here, a major clinical illness, such as lupus erythematosus, rheumatoid arthritis, the nephrotic syndrome, etc., might have compromised phagocytic function as much as the glucocorticoid that was administered.

Secondly, we employed only white blood cells from the peripheral circulation that had not been subjected to the usual sequence of events that precedes their mobilization for host defense functions extravascularly, namely, margination and diapedesis. It is possible, although the likelihood is entirely unknown at present, that the changes concerned with margination and adhesion of white blood cells to vascular endothelium leads to or is associated with a subtle modification of metabolism. If this possibility is real, and Rowley and Whitby (1959) reported that exudate cells from mice were less efficient in vitro and perhaps otherwise altered, then the phagocytic and bactericidal vigor of human exudate cells should be examined before concluding that glucocorticoids are without effect on such essential activities in the host defense reaction. This possible objection does not seem a strong one, since Wood et al. (1951) demonstrated clearly that PMNG played a dynamic and vital part in the clearing of pathogenic bacteria from the circulation of rabbits by means seemingly identical to that relied upon by extravascular phagocytes (Wood, 1953).

A third point worthy of note concerns the procedure used for recovering PMNG from whole blood. Reasonably prompt sedimentation of erythrocytes was brought about by introducing a small amount of clinical dextran to anticoagulated whole blood according to the method of Hirsch and Church (1960). Strauss and Stetson (1960) found that certain bacterial lipopolysaccharides

substantially increased oxygen consumption of white blood cells suspended in whole blood; presumably ingestion of the large molecule stimulated metabolism, since a similar reaction was encountered when inert latex particles were used. Crude dextran, like many other bacterial polysaccharides, not only injures cells but is also pyrogenic, although the means for this noxious effect are unknown. It is possible that the smaller clinical dextran is completely free from such properties, although careful manometric studies of cell metabolism of the sort by Strauss and Stetson (1960) required to eliminate this possibility have not been reported. There is, furthermore, no information to the authors' knowledge as to whether or not clinical dextran of small molecular size is ingested in sufficient quantity or rate to modify subsequently the efficiency of PMNG during phagocytosis and bacterial killing. We assumed that clinical dextran was not injurious to PMNG, and, even so, all but a very small amount was eliminated from the final system during the washing procedure. Other means for the rapid separation of PMNG from whole blood were evaluated, but only this procedure succeeded in eliminating platelets that otherwise contaminated all other preparations.

A final point of concern may be directed toward the lack of data in this system that relate to other microorganisms, since it is known that the pneumococcus is extremely fragile and prone to early autolytic change. Although other organisms such as staphylococci and mycobacteria, for example, may be more resistant to intracellular killing, they were not employed since it was felt that use of a highly susceptible indicator strain would be more likely to reveal the subtle evidence of a metabolic disturbance induced by treatment with glucocorticoid.

Martin et al. (1954) found several years ago that the carbohydrate metabolism of human leukocytes was modified by glucocorticoids, whereas respiration was not altered. More recently, Weissmann and Thomas (1963) suggested that adrenal steroids stabilized cytoplasmic lysosomes recovered from liver of rats. Hence, it is tempting to speculate that the cytoplasmic granules or lysosomes of PMNG, reservoirs of potentially bactericidal enzymes (Cohn and Hirsch, 1960), may be stabilized similarly and thereby be not as freely available for bacterial killing. Until this eventuality has been tested, one cannot exclude finally the possibility that phagocytic bactericidal function can be compromised by adrenal glucocorticoids. Considerable circumstantial evidence, both experimental and clinical, has been cited (Clawson and Nerenberg, 1953; Frenkel, 1960) to suggest that phagocytes

function suboptimally upon extensive exposure of the host to glucocorticoids. One must conclude that additional experimentation with human donors is indicated, with particular emphasis placed upon the influence of moderate steroid administered for protracted periods to otherwise healthy subjects. Only in this way can the variable of complicating disease be eliminated.

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