Effect of Extracts from Rabbit Leukocytes on Levels of Acute Phase Globulins in Rat Serum (35217)

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For some time this laboratory has been engaged in a search for endogenous factors that might be associated with various abnormalities observed in chronic disorders, such as cancer, infection, or rheumatoid arthritis. Recent investigations have indicated that a group of low molecular weight (10,000–20,000) proteins produced by rat or rabbit polymorphonuclear (PMN) leukocytes was involved with the depression of plasma iron (1, 2) and plasma zinc (3). Since this group of proteins was obtained by procedures used to prepare rabbit leukocytic pyrogen, the rabbit preparations exhibited endogenous pyrogen activity as well (2, 4).

A long-known characteristic of chronic disorders is the change in the electrophoretic profile of serum proteins. This change includes the appearance in the rat of a new protein designated α_2-acute phase (AP) globulin (5), as well as an increase in a normally occurring protein designated α_1-AP globulin (6, 7). These AP globulins also appear in the serum in model animal systems designed to study the inflammatory state, *e.g.*, following the injection of endotoxin or turpentine (5, 8, 9). These same model systems also induce fever (10) and lower plasma iron and zinc levels (3, 11, 12).

It appeared pertinent, therefore, to determine whether the group of proteins produced by rabbit PMN leukocytes might also have the ability to stimulate the appearance of AP globulins in the serum of rats. The present experiments are reported in evidence of the role of leukocytes as intermediaries in this facet of acute and chronic disorders.

Materials and Methods. Animals. Inbred male Fischer rats weighing approximately 300 g were fed Purina Lab Chow and water *ad libitum*. They were housed in quarters at 70 ± 2°F with 12 hr of light and 12 hr of dark. The rabbits used were white, mixed breed, and purchased locally; they were fed Purina Rabbit Pellets and water *ad libitum*.

Leukocytic extracts. The methods for induction and collection of PMN leukocytes from rabbits, as well as the preparation of extracts from them have been described (1). In some cases a portion of the leukocytic extract was partially purified by a butanol–methanol method (2). Prior to use in these experiments, each extract was determined to be free of bacterial contamination, to be capable of lowering plasma iron in rats, and to cause a febrile response in rabbits made refractory to endotoxin (1). Additionally, an inactive (by above criteria) control leukocytic preparation was obtained by subjecting a portion of the leukocytes to homogenization at 4° in the same medium used to incubate the remaining portion. This homogenate was given a low speed centrifugation at 4° to remove whole cells, nuclei, and debris; the supernatant solution served as the control extract. Both control and active extracts were adjusted in volume so that each ml represented the material obtained from 2×10^8 cells. Assay by the method of Lowry *et al.* (13) showed both types of extract to contain similar concentrations of protein (3.3–4.0 mg/ml).

Experimental regimen. Two groups of six rats each were given ip injections of 2 ml of active extract ($\approx 4 \times 10^8$ cells) from two different batches of rabbit PMN leukocytes. A third group of six rats received ip injections of 2 ml of control extract ($\approx 4 \times 10^8$ cells). A fourth group of animals received various lower doses of either the usual or the partially purified leukocytic extract. The rats were bled 10 or 48 hr later by cardiac puncture under Seconal anesthesia. The sera were

prepared and stored frozen until assay for AP globulins.

Antisera. The purification of rat α_2-AP globulin and the preparation of its antiserum were accomplished essentially as described by Sarcione (14). Rat serum prepared 48 hr after injection of 0.5 ml of oil of turpentine into the *rectus femoris* served as the starting material for ammonium sulfate fractionation. The precipitated proteins were dissolved in elution buffer (0.15 *M* NaCl, 0.035 *M* Na_2HPO_4, 0.006 *M* NaH_2PO_4, pH 7.3) and applied directly to a column of Sephadex G-200 (2.5 × 40 cm) without prior dialysis. Elution of the column was carried out at a flow rate of 13–15 ml/hr and with collection of 10-ml fractions. The first 10-ml fraction at the void volume contained most of the initial protein peak and was concentrated to 8.25 mg of protein/ml with Lyphogel (Gelman). This concentrate was emulsified (1:1) in Freund's complete adjuvant (Difco). Rabbits were immunized with the above mixture by subcutaneous injection into multiple depots on the back. The initial injection of 1-ml total volume was followed 2 weeks later by injection of 0.5 ml. Two weeks after the second injection the rabbits were bled via the ear vein, and the antiserum was absorbed with normal rat serum at a 1:1 ratio.

The antiserum to α_1-AP globulin was very generously supplied by Dr. D. A. Darcy of the Chester Beatty Research Institute, London, England.

Immunoelectrophoresis. Immunoelectrophoretic runs were made in agar on glass slides using the apparatus of LKB Instruments, Inc. Prepackaged LKB Veronal buffer salts, ionic strength 0.1, pH 8.6, were used in the electrode vessels. Ionagar No. 2 (Consolidated Laboratories, Inc.) was prepared at 1% concentration in the buffer–water–1:1000 Merthiolate solution in the volume proportions of 25:65:10. The appropriate sera were first electrophoresed for 1 hr at 200 V, the trough gel was then removed and diffusion of the antiserum and antigen was allowed to proceed for 48 hr at room temperature in a humid chamber.

Immunodiffusion assays. Rat serum AP globulins were detected by Ouchterlony-type double diffusion in agar. One percent Ionagar No. 2 plus 0.1% sodium azide was prepared in the saline–phosphate buffer as described for elution of Sephadex G-200. The warm agar solution was pipetted (6.6 ml) into flat-bottomed plastic petri dishes (Falcon Plastics, No. 1007, 60 × 15 mm) to a depth of approximately 3 mm. After solidification the agar plates were stored at 4° at least 1 day before using. Wells, 7 mm in diameter, were cut in the plates using a commerical die (Grafar Corp., Auto-Gel) at a separation of 10 mm. Absorbed specific antiserum and appropriate test sera were pipetted (0.09 ml) into the wells, and diffusion was carried out for 48–72 hr at 37° in a humid chamber. The plates were photographed to form a permanent record for analysis of the resulting precipitin bands.

Quantitation of the serum levels of α_1-AP globulin on these plates was performed by the method of Darcy (15, 16). The sera from six normal rats were pooled to form a standard solution of α_1-AP globulin which was arbitrarily assigned a concentration of 100 units/ml and was used to develop a standard curve relating concentration of the AP protein to position of the precipitin band. Each dilution of the standard normal sera was run at least six times in different agar plates. Usually two dilutions of unknown sera were each run four times in different plates. The measurements of the precipitin band positions were made from photographs to the nearest 0.1 mm. The average position of the precipitin band was used to determine the α_1-AP globulin concentration from the standard curve.

Results. To ascertain that the two antisera used in these experiments were monospecific for their respective AP globulins, immunoelectrophoretic studies were made. The results are shown in Fig. 1A. Both of the antisera give only one precipitin arc with serum containing the AP globulins, and the arcs appear in electrophoretically distinct positions. As expected, the α_2-AP globulin was not found in normal rat serum, whereas the α_1-AP globulin is present in the normal serum, although at a lower concentration than that seen in the serum from the turpentine-treated rat.

The ability of the leukocytic extracts to stimulate the appearance of α_2-AP globulin in

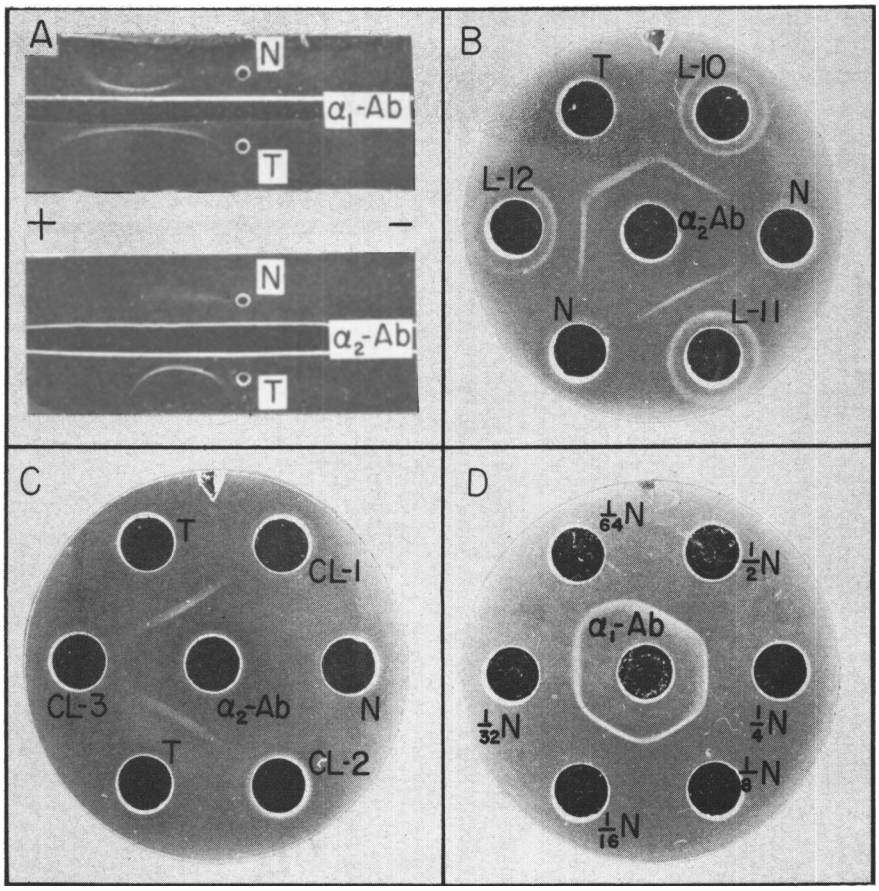

FIG. 1A. Immunoelectrophoresis of sera from normal, N, and turpentine-treated, T, rats versus antisera to α_1- and α_2-AP globulins, α_1-Ab and α_2-Ab. (B) Immunodiffusion showing presence of α_2-AP globulin in sera from rats treated with leukocytic extract, L, or turpentine. (C) Immunodiffusion showing absence of α_2-AP globulin in sera from rats treated with control leukocytic extract, CL. (D) Quantitative immunodiffusion showing change in position of α_1-AP globulin precipitin bands upon serial dilution of serum from a normal rat.

the serum of rats was tested by immunodiffusion of such sera against the absorbed specific antiserum. Photographs of typical test results are shown in Fig. 1B for animals injected with the active extract and in Fig. 1C for animals injected with the control extract. Each immunodiffusion test included known positive and negative sera, from turpentine-treated and normal rats, respectively. A single precipitin band formed for each positive serum and joined adjacent bands in a reaction of identity. The results of a series of such tests are given in Table I. Nine of the 12 rats injected with extracts from 4×10^8 leukocytes gave positive results for the

TABLE I. Immunoassay for α_2-AP Globulin: Precipitin Band Formation by Sera from Male Fischer Rats 48 hr After ip Injection of Extracts from 4×10^8 Rabbit Polymorphonuclear Leukocytes.

		No. of rats responding	
Group	Type of extract	Positive	Negative
1	Leukocytic (batch no. 1)	4	2
2	Leukocytic (batch no. 2)	5	1
3	Control leukocytic	0	6

TABLE II. Immunoassay for α_2-AP Globulin: Precipitin Band Formation by Sera from Male Fischer Rats 10 hr After ip Injection of Extracts from the Indicated Number of Rabbit Polymorphonuclear Leukocytes.

Type of extract	No. of leukocytes	No. of rats responding	
		Positive	Negative
Leukocytic (batch no. 3)	2×10^8	3	0
	1×10^8	3	0
	1×10^7	3	0
Purified leukocytic	2×10^8	3	0
	1×10^8	3	0
	1×10^7	3	0

presence of α_2-AP globulin in their sera 48 hr later; no precipitin bands were evident upon immunodiffusion for the remaining three animals. Immunodiffusion gave negative results for serum α_2-AP globulin in all rats injected with the control leukocytic extract. Table II presents evidence for retention of activity upon partial purification of the leukocytic extract and shows that doses from as few as 1 $\times$ 10^7 cells caused detectable levels of α_2-AP globulin by 10 hr after injection into the rats.

The sera from those animals given extracts from 4 $\times$ 10^8 leukocytes were analyzed for levels of α_1-AP globulin by quantitative immunodiffusion as described for this protein by Darcy. Figure 1D illustrates the variation in precipitin band position with change in concentration upon which the quantitation is based. The results of the assays, Fig. 2, show nearly a twofold increase in the serum α_1-AP globulin in 9 of the 12 rats injected with the leukocytic extract. In contrast, the other three rats had α_1-AP globulin levels below normal. It should be emphasized that these three animals are the same that gave negative responses for the α_2-AP globulin. All of the rats treated with the control leukocytic extract had serum levels of α_1-AP globulin near that of the pooled sera from normal rats used to establish the standard curve for the quantitative assay.

Discussion. The positive responses observed in most of the rats treated with the leukocytic extract indicate that the PMN leukocyte potentially could act as an intermediary in the appearance of serum AP globulins under a wide variety of initial stimuli. The reason for lack of a positive response in three of the rats is not readily apparent. It would appear to be related to a peculiarity of the individual rats, since the α_1-AP globulin levels were below normal and the same leukocytic extract was active in other animals. The homogenization used in the preparation of the control extract provided low levels of leukocyte rupture as indicated by the low protein content of the extract. Additionally, precautions were taken to avoid endogenous protein production in the control extract by keeping it cold and preparing it rapidly. Other investigators have shown that under these conditions only low levels of endogenous pyrogen are produced (17). The amount of this control extract injected (less than 8 mg of protein/rat) was insufficient to elicit a pyrogenic response in rabbits and did not stimulate AP globulins in the rat.

Darcy recently reported on the effects of PMN cells on rat α_1-AP globulin (18). Intact

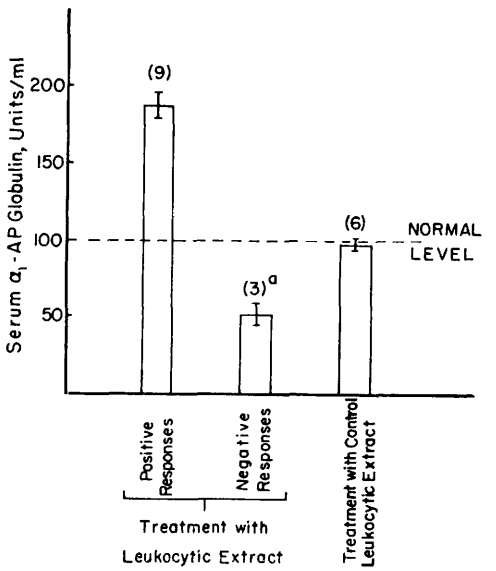

FIG. 2. Serum levels of α_1-AP globulin in rats 48 hr after ip injection of extracts from 4 $\times$ 10^8 rabbit PMN leukocytes. Levels shown are the mean $\pm$ SE for the number of animals shown in parentheses.

[a] These are the same three rats whose sera were negative for α_2-AP globulin (Table I).

cells, or subcellular fractions, from both rat and rabbit leukocytes were used as the stimulating agents. He observed moderate increases in serum α_1-AP globulin when whole cells or microsomal or supernatant fractions were injected. It seems possible that the leukocytes may have been producing some of the endogenous protein during Darcy's preparative procedures. Additionally, the relatively large amount of material injected (up to 200 mg/rat) would enhance the ability to detect low levels of activity. In any case his results, and those reported here, are consistant with the hypothesis that a protein synthesized and excreted by the leukocyte causes the appearance of AP globulins in serum. Direct comparison of the units used here and those reported by Darcy are not possible, since different α_1-AP globulin standards were employed in each case.

The dose of the active extract (from 4×10^8 leukocytes) used in the initial investigations was selected to optimize the potential response. The detection of α_2-AP globulin following treatment with lower doses (Table II) indicated that this response was at least as sensitive as the pyrogenic and plasma iron and zinc responses (1, 3). Since the purification of the extract yields approximately a 10-fold reduction in total protein content, it seems that only a few micrograms of the active protein are necessary to elicit the appearance of α_2-AP globulin in the serum.

The partially purified extract from rabbit PMN leukocytes has been shown to consist of at least six electrophoretically distinct proteins, only one of which provides the pyrogenic activity (19). Whether this single protein is responsible for all of the activities thus far attributable to these leukocytic extracts, or whether each activity is mediated by different protein species, remains to be determined. Leukocytic pyrogen is thought to act upon the hypothalamus in temperature regulation (10). Synthesis of α_2-AP globulin has been demonstrated to occur in some types of tumor tissue (20), as well as in the liver of animals following tumor transplantation or during acute inflammatory reaction to injury (14, 21). The depression of plasma iron levels in tumor-bearers or following endotoxin administration may result from a derangement in iron metabolism in the reticuloendothelial system (11). It would seem, therefore, most logical to proceed upon the hypothesis that a different protein intermediate is responsible for each of the activities ascribed to the leukocytic extract, and that each intermediate has its own specific target organ as a site of action.

Summary. Extracts were prepared from rabbit polymorphonuclear leukocytes by procedures previously shown to yield activity for pyrogenicity and lowering of plasma iron and zinc levels. Rats were injected ip with these extracts, and 10 to 48 hr later their serum was assayed for acute phase globulins by immunological techniques. Positive responses were observed for α_1- and α_2-AP globulins; whereas responses to injections of control extracts were uniformly negative. The data were interpreted to indicate that one or more proteins synthesized and excreted by leukocytes can act as humoral intermediates in the stimulation of serum acute phase globulins during a variety of acute and chronic disorders.

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