Quantitation of Plasma α_2-AP Globulin Before and After Stimulation with Leukocytic Extracts (36187)

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Recent investigations in this laboratory indicated that a group of low molecular weight proteins produced by polymorphonuclear (PMN) leukocytes stimulated the appearance of acute phase (AP) globulins in the serum of rats (1). This group of proteins also was active in the depression of plasma iron and zinc levels (2, 3) and includes the better known leukocytic pyrogen (4). It was postulated that these leukocytic proteins behave as humoral regulators of the cited biochemical abnormalities as observed under a variety of acute and chronic disorders.

It would be of particular value to learn which of the proteins present in the leukocytic extracts are responsible for the various biochemical abnormalities observed following their administration. To adequately evaluate such multiple responses in individual animals, it was necessary to know in some detail the time- and dose-response relationships for each activity. The experiments reported here provide this data for the stimulation of α_2-AP globulin in the rat. Included are details of a procedure developed for the assay of α_2-AP globulin by quantitative radial immunodiffusion. The sensitivity of this assay allows us to report here for the first time the presence, at low levels, of this "abnormal" serum globulin in normal rats.

Materials and Methods. Female Holtzman rats weighing approximately 200 g were fed Purina Lab Chow and water *ad lib.* They were housed in quarters of 70 ± 2°F with 12 hr of light and 12 hr of dark. The rabbits used were of mixed breed and were purchased locally; they were fed Purina Rabbit Pellets and water *ad lib.* The method of preparation of crude or partially purified extracts from PMN leukocytes, including controls against bacterial contamination, has been described (1). The method of preparation and specificity of the α_2-AP globulin antisera (α_2-Ab) were also previously reported (1).

Rats were injected ip with appropriate leukocytic extracts at various doses ranging from 1×10^5 to 2×10^8 (expressed as the number of leukocytes from which the extract was obtained). The time- and dose-response experiments were all performed using the same batch of crude extract from rabbit PMNs. At various time intervals after injection, the rats were bled and their plasma prepared. If an animal was to be bled only once, the blood was obtained under seconal anesthesia by cardiac puncture with a heparinized syringe. If an animal was to be bled several times to obtain a time-response profile, the blood was collected under light ether anesthesia from the tail in heparinized capillary tubes. Unless assayed for α_2-AP globulin immediately, the plasma was stored frozen until assay was convenient.

Plasma levels of α_2-AP globulin were quantitated by use of radial immunodiffusion. The general concepts and techniques followed were based on those reported for the quantitation of serum immunoglobulins (5). Saline–phosphate buffer (0.15 *M* NaCl, 0.035 *M* Na_2HPO_4, 0.006 *M* NaH_2PO_4, pH 7.3) was made 0.1% in sodium azide and used to prepare 1% (by volume) α_2-Ab and 2% (by weight) Ionagar No. 2 (Consolidated Laboratories, Inc.) solutions. These were then mixed at a 1:1 ratio at 50°, and 13 ml poured onto a $3\frac{1}{4} \times 4$ in. glass plate (lantern slide cover glass). The gelled antibody–agar plates were stored in a humid chamber at 4° until use. Forty-eight wells were cut in each plate (rectangular grid of 6×8 wells) with a 15-gauge syringe needle which had been sharpened after removal of the point. Rat serum, prepared 48 hr after injection of 0.5 ml of oil of turpentine into the *rectus*

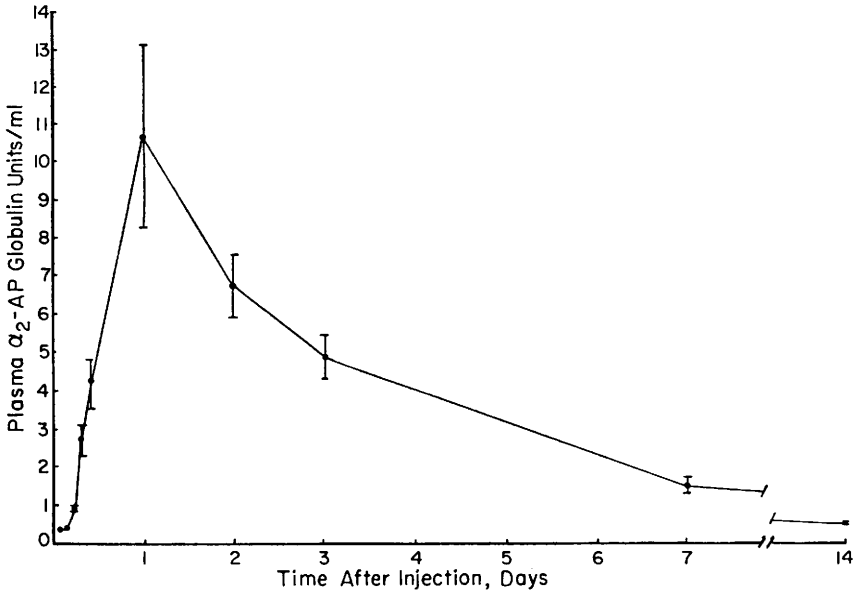

FIG. 1. Levels of α_2-AP globulin in the plasma of rats following ip injection of crude extracts from 1 × 10^8 rabbit PMN leukocytes. Levels shown are the mean ± standard error for 15 animals per point.

femoris, served as a standard source of α_2-AP globulin which was arbitrarily assigned a concentration of 100 units/ml. Appropriate dilutions of the α_2-AP globulin standard serum and plasmas to be assayed were placed in the wells of the antibody–agar plates. Preliminary trials established the appropriate amount of α_2-Ab to be used in the agar as well as the concentration range of our standard to be used: 1/32, 1/64, 1/128, and 1/256 dilutions of the standard were employed. Thus, the 48 wells available allowed the four concentrations of standard and up to 20 unknowns to be assayed in duplicate on each antibody–agar plate. Radial immunodiffusion was allowed to proceed in a humid chamber at room temperature for 24 hr. The assay plates were then photographed to form a permanent record of the results. The diameters of the rings of antigen–antibody precipitate were measured from enlarged photographs to the nearest 0.1 mm. A standard curve was prepared for each assay plate relating ring diameter to α_2-AP globulin concentration, and the concentration of the unknowns was read from this curve.

Preliminary qualitative assays for α_2-AP globulin Ouchterlony-type double diffusion in agar were found to be helpful in estimating the appropriate dilution of a plasma for subsequent quantitation. For the qualitative assays 1% Ionagar No. 2 (in azide buffer used for quantitation) was poured onto microscope slides (1 ml/1 in.^{2}). Three sets of one center and four peripheral wells (3 mm separation) were cut on each slide with the 15-gauge needle. α_2-Ab was placed in the center wells and the unknown plasmas in the peripheral wells. Diffusion was allowed to proceed in a humid chamber at room temperature for 15–24 hr. The resulting precipitin bands were graded as negative, weak, medium, or strong as compared to a known standard α_2-AP globulin source.

Results. To determine the time sequence of the plasma α_2-AP globulin response, rats were injected ip with crude extract from 1 × 10^8 rabbit leukocytes. The animals were bled from the tail at 2-hr intervals up to 10 hr and at 1, 2, 3, 7, and 14 days after injection. The plasma was assayed by radial immunodiffusion, and the results are shown in Fig. 1. No change was found in the α_2-AP globulin level during the first 4 hr following injection. Beginning with the 6-hr determination, the α_2-AP globulin level rises sharply reaching a maximum of 10.7 units/ml at 24 hr. The

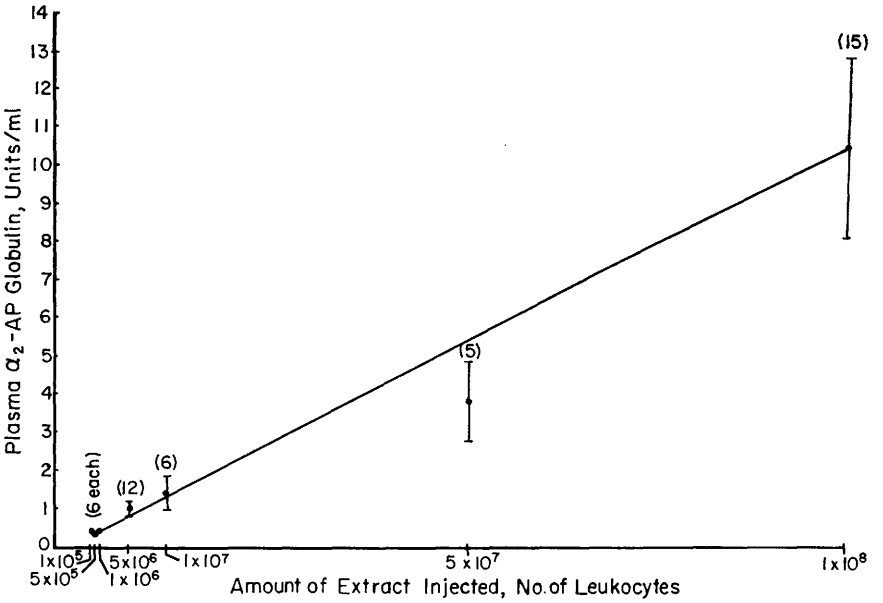

FIG. 2. Levels of α_2-AP globulin in the plasma of rats 24 hr after ip injection of crude extracts from the indicated number of rabbit PMN leukocytes. Levels shown are the mean ± standard error for the number of animals shown in parentheses.

plasma levels then gradually declined reaching normal by 14 days after injection. To insure that the stress of repeated tail bleeding did not contribute to the response, three normal rats were subjected to the same series of bleedings up to the 24-hr point. These plasmas were assayed for α_2-globulin by the qualitative immunodiffusion procedure and found to be uniformly negative. Experience with the qualitative assay has shown that the lower limit for detection of α_2-AP globulin is in the range of 1–2 units/ml.

The dose-response of rats to the crude rabbit leukocytic extract was assessed by ip injection of doses ranging from 1×10^5–1×10^8. All animals were bled by cardiac puncture 24 hr later, and the plasma was assayed by radial immunodiffusion. The results are shown in Fig. 2. Doses at or below that obtained from 1×10^6 leukocytes did not elevate the plasma α_2-AP globulin. Increasing levels of plasma α_2-AP globulin were observed at doses from 5×10^6 up to 1×10^8 (the maximum tested).

During the development of the quantitative radial immunodiffusion assay, plasma from normal rats consistently yielded small rings of precipitate around the wells corresponding to an α_2-AP globulin concentration of about 0.3–0.4 units/ml. Similar plasma levels were also found at the initial points of the time- and dose-response curves (Figs. 1 and 2). Since α_2-AP globulin had not been observed in normal rats by previous investigators (1, 6, 7), this finding was investigated further. To insure that the low levels of α_2-AP globulin observed were not due to a non-specific reaction of the plasmas with the agar or rabbit antiserum, radial immunodiffusion assay plates were prepared substituting an absorbed (with normal rat serum) normal rabbit serum for the α_2-Ab. Wells in these assay plates were filled with α_2-AP globulin standard serum, normal rat plasma, and rat plasmas previously determined to have elevated α_2-AP globulin levels. None of these samples yielded precipitin rings after 24 hr of diffusion.

It was possible that precipitin rings were observed, because small amounts of antibodies to normal rat serum were not completely removed by the single step of absorption used in preparing the α_2-Ab (1). Therefore, a portion of the α_2-Ab was carried through a second absorption with normal rat serum, and assay plates were prepared utilizing either the usual singly absorbed α_2-Ab or the doubly

absorbed α_2-Ab. The wells in these assay plates were filled with the usual α_2-AP globulin standard serum and plasmas from 10 normal rats. After 24 hr of diffusion, precipitin ring diameters indicated an α_2-AP globulin concentration of 0.38 ± 0.02 and 0.41 ± 0.02 units/ml in the 10 normal plasmas for the singly and doubly absorbed α_2-Ab, respectively. We concluded that α_2-AP globulin occurs in the plasma or serum from normal rats. As further confirmation of this conclusion, plasma or serum from normal rats, or from such animals after various control injections, were assayed α_2-AP globulin levels by radial immunodiffusion. Among 54 animals the α_2-AP globulin level was 0.38 ± 0.01 units/ml; and the maximum and minimum values encountered were 0.55 and 0.29 units/ml, respectively.

All experiments thus far on the stimulation of α_2-AP globulin by leukocytic extracts have employed PMN leukocytes obtained from the peritoneal cavity of rabbits. It was also of interest to determine if extracts from rat leukocytes were active for stimulation of rat α_2-AP globulin, and the results are given in Table I. Approximately 3-fold and 22-fold increases in plasma α_2-AP globulin were observed at the 8- and 15-hr intervals, respectively.

Discussion. The first detectible rise in plasma α_2-AP globulin at 6 hr after stimulation with PMN extracts (Fig. 1) is 2 hr earlier than that reported by Weimer and Benjamin (6) following the injection of turpentine. Peak levels in both situations were attained around 24 hr. Turpentine results in more prolonged stimulation, since the high level is maintained longer than 48 hr whereas the stimulation with leukocytic extracts declines after 24 hr. These results are consistent with the hypothesis that the leukocytic protein acts as a relatively short-lived humoral intermediate. The gradual decline of plasma α_2-AP globulin over a 2-week period in both circumstances probably reflects the biological life of the α_2-AP globulin once formed.

TABLE I. Plasma Levels of α_2-AP Globulin after Treatment with Crude Extracts from Rat PMN Leukocytes.

Time after injection (hr)	No. of animals	α_2-AP globulin units/ml
Controls	54	0.38 ± 0.01[a]
8[b]	11	1.08 ± 0.18
15[b]	2	8.69

[a] Mean ± standard error.

[b] Extract from 2 × 10^8 leukocytes injected ip (4 different batches at 8 hr; 1 at 15 hr).

Rabbit leukocytic extracts cause an initial temperature elevation in rabbits and depression of plasma iron and zinc in rats around 15 min and 2–4 hr after administration, respectively (2, 3, 9). The longer time required to see elevation of α_2-AP globulin may reflect the greater time required to set new protein synthesis in motion as opposed to the time required to block a preexisting metabolic path in the case of temperature or iron regulation. The depression of plasma iron and zinc by the leukocytic extract reaches a maximum around 12 and 8 hr after treatment, respectively. The increase of α_2-AP globulin does occur rapidly enough, therefore, to allow comparison with the alterations in iron and zinc metabolism in the same animal at about 8 hr after stimulation.

The dose-response (Fig. 2) of α_2-AP globulin to the leukocytic extract compares very favorably with that reported for the depression of plasma iron (2). In both cases the minimum dose to produce measurable effects required extract obtained from 1 × 10^6 leukocytes. Again these results permitted measurement of both activities simultaneously in the same animal at a single dose level.

Previous studies of α_2-AP globulin indicated its presence in fetal, neonatal, pregnant, injured, or diseased rats but not in normal rats (1, 6, 7). Sarcione (10), in quantitating α_2-AP globulin by an isotope incorporation procedure, found low levels of radioactivity in samples isolated from normal rats, but coprecipitation of other labeled proteins could not be ruled out. Since such a wide variety of circumstances or stresses can elicit its stimulation, and it is relatively long-lived, it is not too surprising to find α_2-AP globulin present at very low levels in normal animals. Its detection only awaited a sensitive assay procedure. The significance of its presence remains to

be determined, however.

The quantitative radial immunodiffusion procedure developed for the assay of α_2-AP globulin not only has high sensitivity and specificity, but also is relatively simple in terms of technique or materials needed. We have experienced close agreement between assays of the same samples on separate plates including stability in the size of the precipitin rings obtained with the various dilutions of the standard α_2-AP globulin serum. Since the standard serum has been used over a period of several months, and some samples have been reassayed several days apart, the α_2-AP globulin is apparently highly stable if stored frozen. Limitations of the procedure include the length of time between start and completion of the assay (about 3 days) and the present lack of a highly pure α_2-AP globulin as an absolute standard.

The demonstration of the activity of extracts from both rat and rabbit leukocytes in the stimulation of plasma α_2-AP globulin is in agreement with related plasma iron investigations (2, 4), and strengthens the argument for their role as humoral intermediates in the regulation of several biochemical abnormalities associated with acute and chronic disorders. The data now available permits closer investigation of the relation between such abnormalities and the several proteins present in the leukocytic extracts (11). One approach is the testing of electrophoretic fractions from the leukocytic extracts for biological activity, and such experiments are presently underway in this laboratory.

Summary. A procedure developed for the quantitation of acute phase globulin by radial immunodiffusion was found to be sensitive enough to detect low levels of the "abnormal" protein in the plasma of normal rats. Time- and dose-response curves for the stimulation of plasma acute phase globulin in the rat by crude extracts from rabbit polymorphonuclear leukocytes were determined. The results were compared with previous investigations of the effects of such extracts on body temperature and plasma iron and zinc levels. Extracts from rat leukocytes were also found to be active in the stimulation of acute phase globulin. The data were found to be consistent with the hypothesis that leukocytes produce one or more humoral intermediates that regulate several biochemical abnormalities associated with acute and chronic disorders.

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