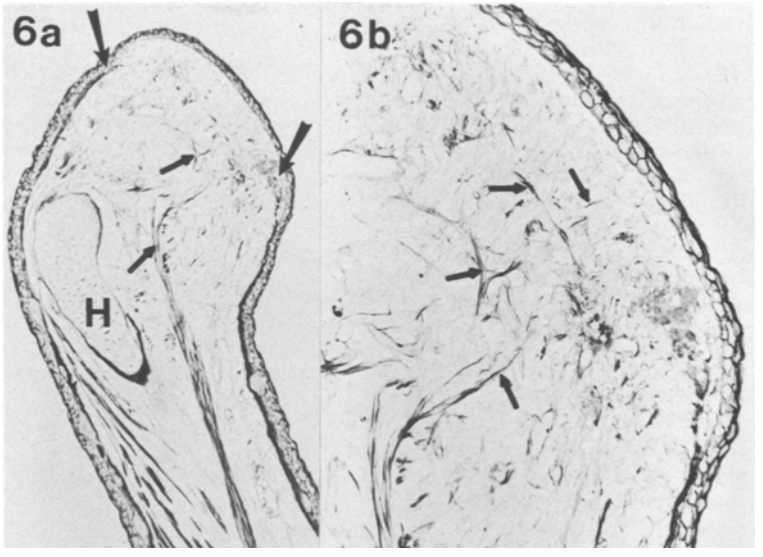

Fig. 6. Micrographs of a longitudinal section stained for nerves through a left forelimb denervated 1 day post-amputation and fixed 14 days post-amputation. The section in *a* is slightly lateral to the radius and ulna. Abundant nerve fibres [small arrows] in the area of dedifferentiation are correlated with an increase in mitosis at this time. The area of wound epidermis is somewhat restricted by the edges of the dermis [large arrows]. H, humerus. Samuel's nerve stain. *b* is a higher magnification of a portion of the section in *a*. a, × 40. b, × 100.

3.5% maximum of control blastemas is not known. The regeneration time for left limbs, days 13–22, was the same as for controls, days 3–12 (figure 1).

Denervated limbs showed movement on days 10 and 11 and sensitivity to touch on days 12, 13, and 14, suggesting that nerves were at the tip of the limb at the time mitosis began. Nerve staining confirmed this view. Those limbs on days 12, 13, and 14 which showed increases in the mitotic index also had abundant nerve fibres coursing among the dedifferentiated cells in the distal region of the limb (figure 6). Most important, ultrastructural examinations of denervated limbs at the time of re-innervation, during the 12 to 14 day period established in this study, will make it possible to test whether nerves make contact with dedifferentiated cells before cycling can begin.

1 This work was supported by grant PCM 76-11807 from the National Science Foundation.
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Inhibition of neutrophil-mediated cytotoxicity by α2 macroglobulin[1]

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Summary. Highly purified human α2 macroglobulin (1.2–10 mg/ml) was shown to inhibit phytohemagglutinin-induced or antibody-induced lysis of chicken erythrocytes by polymorphonuclear neutrophils. Inhibition was not associated with impaired contact between effector and target cells but rather with the antiprotease activity of α2 macroglobulin.

Many of the tissue lesions in Arthus reactions are mediated by lysosomal enzymes, particularly acidic proteases, released by polymorphonuclear (PMN) neutrophils upon ingesting antigen-antibody complexes. In the absence of phagocytosis, PMN which encounter immune complexes deposited upon a solid matrix extrude lysosomal enzymes by a mechanism of reverse endocytosis or frustrated phagocytosis[2]. 2 in vitro models have been designed to investigate the mechanisms of these reactions. Basically they use non-phagocytable ^{51}Chromium-labelled target cells, e.g. chicken erythrocytes, which are incubated with PMN. Precise measurement of target cell lysis is achieved by determination of ^{51}Cr-release into the supernatant. The cytotoxic reaction is triggered either by agglutinating lectins[3] or by anti-target cell IgG antibodies[4] which bind to PMN surface receptors[5]. Little is known of the various mechanisms which may control the in vivo counter-parts of such cytotoxic reactions. We have investigated the possible regulatory role of α2 macroglobulin (α2 M), one of the major plasma protease inhibitors, the serum levels of which are known to rise during inflammatory reactions.

Material and methods. Human PMN were obtained from normal donors. Leucocytes were separated by sedimentation of heparinized blood on Dextran (Pharmacia Fine Chemicals, 5.04% solution, 24 vol.) - Isopaque (Winthrop, 4 vol.) followed by centrifugation on Ficoll-Isopaque as already described. PMN were collected in the pellet; they were freed of erythrocytes by hypotonic lysis, then washed and resuspended in RPMI 1640 medium supplemented with antibiotics and 2% fetal calf serum. Chicken red blood cells (CRBC) obtained from white Leghorn, 2–6-month-old, were labelled with ^{51}Chromium ($NA_2\ ^{51}CrO_4$, C.E.A. Gif-sur-Yvette) as described elsewhere[6]. Highly purified fractions of α2 M were obtained from Drs Bonneau and Latour (Institut Mérieux, Marcy l'Etoile). They were prepared from pooled human plasma by Rivanol precipitation

and ion-exchange chromatography. The purity of the α2 M fraction was checked by immunoelectrophoresis with polyvalent or specific antisera.

The cytotoxic reaction was triggered by the addition of phytohemagglutinin (PHA, Wellcome reagents, 30 µg/ml, final dilution), or rabbit anti-CRBC serum (1:10,000 to 1:250,000, final dilution). The cell mixture (0.2 ml, 20 PMN:1 CRBC) in the presence of PHA or antiserum was distributed into the wells of a plastic plate (Limbro plastics, MF 96). Each test was done in triplicate. At the end of the incubation period, supernatants were recovered using an automatic processor and radioactivity was counted in an autogamma spectrometer (Packard No 5375). Results were expressed first as cytotoxic indices according to the formula

$$CI = \frac{E - S}{100 - S}$$

where E stands for 'experimental' and S for 'spontaneous' release. Results were expressed as percentages calculated from the ratio (CI with inhibitor/CI without inhibitor) $\times$ 100.

Results. a) PHA-induced cytotoxicity. With PHA, maximal CRBC lysis was achieved in 18 h. α2 M was shown to potentiate CRBC lysis at low concentrations (0.6 mg/ml) and to inhibit cytotoxicity at higher concentrations. Both effects were consistently reproduced in 5 experiments; a typical result is shown in figure 1. Complete inhibition was achieved with 2.5–5 mg α2 M/ml, a concentration close to that in normal serum. PMN and CRBC were agglutinated in the presence of PHA; addition of α2 M (0.6–1.2 mg/ml) increased the agglutination. In this concentration α2 M did not prevent ^{51}Cr release from CRBC killed by one cycle of freezing and thawing. The possibility that α2 M may inhibit cytotoxicity by preventing the binding of PHA to PMN was also considered. PHA was labelled with ^{3}H according to Miller and Great[7], and the amount of ^{3}H-PHA bound to 5×10^5 PMN was measured in the presence of α2 M (2.5–10 mg/ml); no significant inhibition of PHA binding was observed. Finally to exclude the possible contribution of small molecules carried by α2 M to cytotoxicity inhibition, the α2 M fraction was extensively dialyzed against buffered saline: the inhibitory capacity remained unchanged.

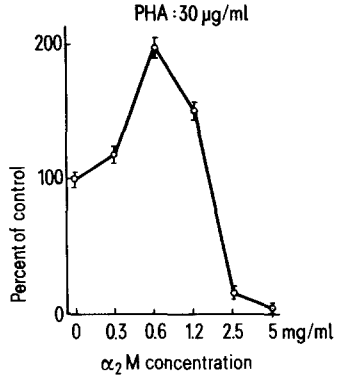

Fig. 1. Differential effect of α2 M on PHA-induced neutrophil cytotoxicity.

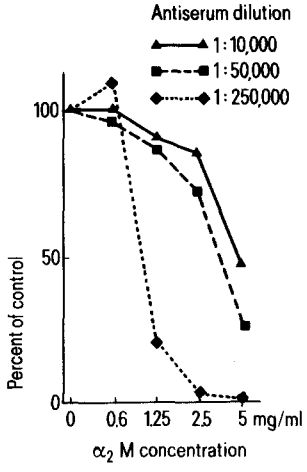

Fig. 2. Inhibitory effect of α2 M on antibody-dependent neutrophil cytotoxicity.

b) Antibody-induced cell cytotoxicity. With anti-CRBC serum, maximal release was obtained within 5 h at 37 °C, no lysis occurring with normal rabbit serum. CI were positively correlated with anti-CRBC serum concentrations (e.g. 70% at 1:10,000; 54% at 1:50,000 and 15% at 1:250,000). Addition of α2 M at concentrations ranging from 0.6 to 40 mg/ml resulted in a dose-dependent inhibition; 50% inhibition was achieved with 5 mg α2 M/ml that is twice the α2 M concentration in normal serum. However, as shown in figure 2, ADCC performed at low antibody concentration was readily inhibited with 2.5 mg α2 M/ml, whereas ADCC at higher antibody concentrations was only partially inhibited with high doses of α2 M. Since ADCC is inhibited by aggregated IgG[8], we checked that inhibition by α2 M was not due to contaminating IgG. After extensive absorption on protein A sepharose, the inhibitory capacity of the α2 M fraction remained unchanged. One mechanism which may account for ADCC inhibition by α2 M is inhibition by steric hindrance of the contact between PMN and CRBC. Though no rosette formation can be detected at the low antiserum concentration used in the ADCC reaction, up to 70% PMN were found to form erythrocyte-antibody (EA) rosettes at maximal subagglutinating antiserum concentration. Addition of 1.2–10 mg α2 M/ml did not interfere with EA-rosette formation, suggesting that steric hindrance was not the main mechanism of ADCC inhibition.

Discussion. These results demonstrate that human α2 M inhibits PMN-mediated lysis of heterologous erythrocytes. Inhibition can be attributed to α2 M itself because of the high purity of the α2 M fractions tested, and the lack of evidence for a possible contribution of contaminants such as IgG or dialysable substances. The precise mechanism(s) of inhibition remain(s) to be investigated. So far, α2 M does not appear to interfere with the initial step of any of the reactions studied: neither the binding of PHA onto PMN, nor the contact between PMN and erythrocyte-antibody complexes were found to be impaired. Other inhibitory mechanisms may be considered in view of the broad spectrum of α2 M antiprotease activity. The protein was shown to form complexes without involvement of the active site of the enzymes as the esterase activity of bound enzymes remained intact, although larger molecules such as the natural protein substrates could no longer be digested[9]. Proteolytic enzymes are present not only in PMN granules but also at the cell surface, as shown by the fixation of natural antiproteases such as trasylol[10]. These enzymes are likely to play an important role in the development of tissue lesions induced by PMN, as in the 2 in vitro cytotoxic reactions which can be inhibited by α2 M at concentrations slightly higher than that of normal serum. These findings will stimulate further in vivo studies to see whether α2 M might prove useful for the control of Arthus-like reactions.

1 This study was supported by D.G.R.S.T. contract No 77-7-1381.
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Functional potential of ectopic marrow autotransplants[1]

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Summary. Ectopic autotransplants of the marrow tissue, form within 4 weeks, stable nodules which respond to erythropoietic modulation in a manner similar to other parts of the marrow. The findings indicate that these autotransplants are an integral part of the total hemopoietic mass.

When bits of marrow tissue are autotransplanted to such ectopic sites as s.c. tissue they undergo a well-defined regenerative process, similar to marrow embryogenesis[2,3]. Its end product is a nodule of marrow tissue, surrounded by a shell of bone (figure 1). In rats the regenerative process is completed within 4 weeks and from then on, the ectopic marrow nodule survives as an integral part of the organism. The functional potential of these marrow nodules has not been studied. We now have used stimulation and suppression of erythropoiesis to determine if the cellular content of these nodules responds appropriately to these experimental manipulations. Hypertransfusion and phlebotomy have been used respectively to suppress and to stimulate erythropoiesis.

Materials and methods. Male Wistar rats, 300–400 g, were used. Operations were done under aseptic conditions. For marrow autoimplantation, the knee joint was exposed and an opening was drilled through the articular surface of the femur. A polyethylene tube was driven gently into the marrow cavity and the free end of the tube was then clamped. The tube, now containing the marrow tissue, was then slowly removed. The marrow was implanted in a pocket incised in the s.c. tissue of the abdomen. Hematocrit, and reticulocyte counts were determined twice a week. Animals were killed, 4 weeks after implantation. The implants were removed and Wright-Giemsa-stained smears of the marrow contained within the implants were used for differential counts which were made on 1000 cells. Histologic examination was also made on long bones. 3 groups of animals (at least 10 rats per group) were studied:

Group 1 (hypertransfused). Animals were transfused with 12 ml of fresh blood twice a week beginning the day the implant was made. The amount of blood transfused was adjusted so as to maintain the hematocrit above 65% and the reticulocyte count below 0.1%.

Group 2 (plebotomized). Animals were phlebotomized twice a week and each time 5–7 ml of blood were removed. Attempts were made to maintain the hematocrit around 30% and the reticulocyte counts around 10%.

Group 3 (control). Implants were made but animals were neither phlebotomized nor hypertransfused. The mean hematocrit in this group was 48% and the mean reticulocyte count was 1%.

Results and discussion. The proportion of erythroid cells in all groups, is shown in figure 2. The mean (± SD) was 22.08% (± 11.25), 39.2% (± 13.93) and 3.34% (± 2.82) re-

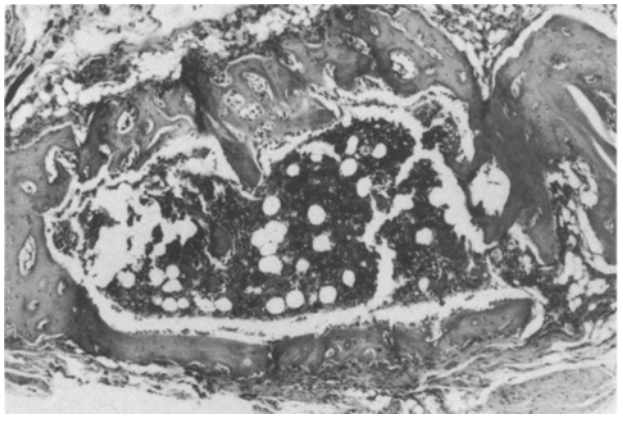

Fig. 1. Histologic section of s.c. marrow nodule in untreated animals showing hemopoietic tissue surrounded by a shell of bone. × 16.

	Hypertransfused	Control	Phlebotomized
Hematocrit %	69.66 (± 5.03)	48.66 (± 0.57)	30 (± 2)
Reticulocyte %	0.0 (± 0.0)	0.83 (± 0.28)	11.50 (± 3.04)

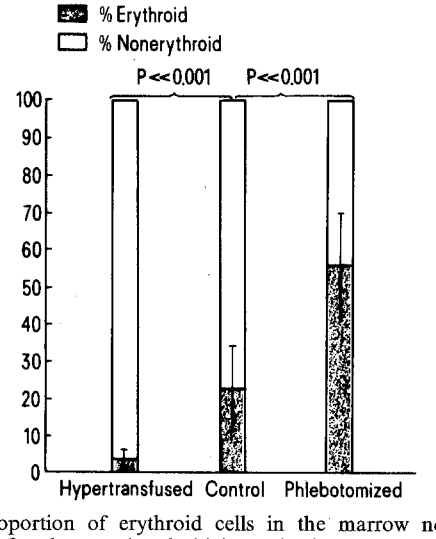

Fig. 2. Proportion of erythroid cells in the marrow nodules of hypertransfused, control and phlebotomized groups. Mean (± SD) hematocrits and reticulocyte counts are also indicated.