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Identification of pregnancy-associated α-macroglobulin on the surface of peripheral blood leucocyte populations

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SUMMARY

Pregnancy-associated α-macroglobulin (PAM) was identified on the surface membranes of human peripheral blood leucocytes by immunofluorescent staining. Subjects having a high serum-level of the glycoprotein (pregnant and contraceptive steroid-treated women) were shown to possess a significantly greater proportion of PAM-positive cells than those (normal males) with a low concentration. However, no correlation was found between the serum values and the percentage of PAM-associated leucocytes. Studies employing purified leucocyte-types and the simultaneous detection of PAM and lymphocyte surface markers indicated that only sub-populations of T lymphocytes (23%) and monocytes (40%) were associated with this serum protein.

INTRODUCTION

Pregnancy-associated α-macroglobulin/α_2-glycoprotein (PAM) is a high-mol. wt. serum protein (Stimson & Eubank-Scott, 1972; Von Schoultz & Stigbrand, 1973), the concentration of which rises dramatically during pregnancy. Variations in PAM-blood levels have also been observed in cancer patients undergoing treatment and it has been shown that the concentration correlates with the course of the disease (Stimson, 1975a).

PAM has been shown to have a marked inhibitory effect on several *in vitro* methods of immunological assessment and it has been suggested that the glycoprotein may assist in reducing the maternal immune response to the foetus (Stimson, 1975b; Than *et al.*, 1975; Damber *et al.*, 1975). Recent studies have indicated that the suppressive activity of PAM depends upon a direct effect exerted on lymphocytes involved primarily in cell-mediated immune responses (Stimson, 1976) and that peripheral blood leucocytes appear to have the capacity to synthesise the α-globulin *in vitro* (Stimson & Blackstock, 1975).

The present investigation was undertaken to confirm the association of PAM with leucocytes and to establish the cell types involved. This was achieved by identifying PAM on the membranes of purified leucocyte types using immunofluorescent staining and by subjecting lymphocytes to the simultaneous detection of the glycoprotein and three surface markers: cells forming rosettes with sheep red blood cells (SRBC) (E-binding lymphocytes) as a measure of T lymphocytes; lymphocytes with surface immunoglobulin (Ig) or with receptors for C3, which formed rosettes with antiserum-treated SRBC and complement (EAC-binding lymphocytes), were regarded as B lymphocytes (Habeshaw & Young, 1975; Holm *et al.*, 1975).

MATERIALS AND METHODS

Peripheral blood samples. Heparinized peripheral venous blood (20 U/ml) was obtained from healthy pregnant women (primigravidae, gestation 37–40 weeks; aged 21–26 years), normal male subjects (aged 22–32 years) and females receiving contraceptive steroids (aged 21–27 years). This was allowed to sediment for 90 min at 37°C and the leucocyte-rich plasma

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separated. The concentration of PAM in the plasma was determined using an enzyme-immunoassay procedure (Stimson & Sinclair, 1974). Leucocytes from pregnant women with PAM levels > 1350 μg/ml were employed in all the experiments carried out to establish the cell types associated with the glycoprotein.

Preparation of leucocytes. Mononuclear cells and granulocytes were isolated from the leucocyte-rich plasma by Ficoll–Isopaque (F.I.) separation (Böyum, 1968), washed twice and resuspended in Medium 199 (Wellcome) containing antibiotics and 20% v/v autologous serum. Granulocytes were also treated with 0·83% ammonium chloride to remove contaminating erythrocytes and this yielded a 98% pure cell population, smears examined with Leishmann's stain. Adherent-cell monolayers were prepared by inoculating 2 ml of the mononuclear cells, adjusted to $1{\cdot}5\times10^7$/ml, into plastic tissue culture dishes (30 mm, Flow Laboratories) and incubated for 2 hr and then 3 hr at 37°C in 5% CO_2 in air (Koller *et al.*, 1973). Non-adherent cells were removed by vigorous washing (x5) with warm medium. Examination of such monolayers showed that > 95% of the cells ingested neutral red dye (Habeshaw & Young, 1975) and that smears consisted of morphologically typical monocytes.

The non-adherent cells (8×10^6/ml) were incubated in a column (1·5 × 8 cm) containing glass beads (0·45–0·50 mm diameter) for 30 min at 37°C. The cells eluted from this column were shown to consist of > 98% lymphocytes by smear staining —and < 1 in 500 ingested neutral red dye. No preferential enrichment of E-rosetting or immunoglobulin-bearing cells was found using this technique.

Antisera. Fluorescein and rhodamine-conjugated goat anti-human immunoglobulin (IgA + IgG + IgM) IgG was purchased from Microbiological Associates. The specificity of the antisera was tested by immuno-electrophoresis against normal human serum. Fluorescein-labelled goat anti-rabbit immunoglobulin (Hoechst Pharmaceuticals) serum was absorbed six times with leucocytes (6×10^6 cells/ml antiserum) from normal male subjects and used in the indirect immunofluorescence procedure. These antisera were employed at a final dilution of 1:12 in phosphate-buffered saline (PBS), pH 7·2.

Rabbit anti-SRBC serum was obtained from Flow Laboratories and rabbit anti-albumin from Hoechst Pharmaceuticals.

The antisera to PAM were produced in rabbits by immunization with PAM, prepared as previously described (Stimson & Eubank-Scott, 1972), mixed with FCA. The IgG fraction of the antisera was prepared by 50% ammonium sulphate precipitation (pH 6·5, 4°C) and column chromatography on DEAE-cellulose (Whatman DE-52). This was then rendered specific for PAM by absorbing out (four times) any contaminating antibodies on cyanogen bromide–Sepharose 4B (Pharmacia) to which serum proteins from 'PAM-free females' had been coupled. The antisera obtained gave only one precipitation line over a range of dilutions against pooled late pregnancy plasma (1500 μg PAM/ml) on two-dimensional immunoelectrophoresis.

Rabbit anti-PAM IgG was conjugated with fluorescein isothiocyanate (isomer I) by the dialysis method of Clark & Shephard (1963). Any molecules overlabelled with fluorescein were removed by treatment of the conjugates with DEAE-cellulose equilibrated with PBS. The molar fluorescein/protein ratio of the conjugates was calculated by determining the extinctions at 495 nm (dye) and 280 nm (protein) and found to vary from 1·9–2·8.

Normal rabbit serum (NRS) was treated in an identical fashion to the anti-PAM and served as a control for all cell preparations. Soluble antigen–antibody complexes were prepared by incubating excess human IgG with sheep anti-human IgG at 37°C for 45 min and then at 4°C for 18 hr.

The $F(ab')_2$ fragment of the anti-PAM was prepared by digesting the IgG with pepsin (twice crystallised-Sigma) in 0·1 M acetate buffer, pH 4·0 at 37°C for 18 hr. The digest was then subjected to gel chromatography on Sephadex G-150, with PBS as the eluting buffer, and the peak containing the moiety was concentrated by ultrafiltration.

All antisera were centrifuged at 100,000 ***g*** for 1 hr immediately prior to use to remove any aggregated material and, where appropriate, contained 2 mg/ml sodium azide.

The fluorescence procedure. In the direct immunofluorescence procedure 5×10^5 washed cells were suspended in 0·2 ml diluted antiserum and incubated for 30 min at room temperature. The stained cells were washed three times in PBS and suspended in 0·1 ml 50% (v/v) glycerol buffered in PBS. Indirect immunofluorescence was carried out by incubating the cells for 30 min in rabbit anti-PAM IgG, washing (three times) and then incubating for 30 min in fluorescein-conjugated goat anti-rabbit immunoglobulin. The cells were finally processed as above.

The stained preparations were examined on a Wild M20 microscope equipped with quartz-iodine and HBO 200 mercury vapour light sources. Fields were viewed simultaneously or consecutively using transmitted light darkground illumination from both sources and the appropriate excitor and barrier filters. The results expressed are based on the average of at least four counts of 150 cells. NRS control values (if any) have been subtracted from these results.

Isopycnic density gradient centrifugation of leucocytes. Linear density gradients of 15–28% w/w bovine serum albumin (BSA) in unbuffered balanced salt solution (BSS) pH 5·1, were prepared according to the method of Shortman (1968) in 14 ml cellulose nitrate centrifuge tubes. Leucocytes ($7\text{–}9\times10^7$) were washed twice and resuspended in 7 ml dense (28% w/w) BSA-BSS prior to generation of the gradient. Centrifugation was carried out in a Beckman Model L2-65B Ultracentrifuge (SW 40 rotor) at 3800 ***g*** for 45 min at 4°C. Six fractions of 2·25 ml were collected and washed three times in chilled PBS or medium 199—fractions were numbered from the top of the gradient. The density of each fraction was determined at 4°C using a linear density gradient prepared with bromobenzene (density 1·49 g/cm^3) and white paraffin (density 0·88 g/cm^3) (Miller & Gasek, 1960), both saturated with BSS. This gradient was calibrated with standard sucrose samples (dissolved in BSS) and 5 μl droplets of these and the fractions were introduced under the meniscus using a microsyringe. The position of each droplet was determined with a travelling microscope.

E-binding lymphocytes. Sheep red blood cells in Alsever's solution (Difco) were washed three times with Hanks' balanced salt solution (HBSS). SRBC, 0·1 ml ($1{\cdot}5\times10^8$) was added to 0·1 ml mononuclear cells (6×10^6/ml) in Medium 199 and

centrifuged (150 *g*, 5 min) at room temperature. After incubation at 4°C for 18 hr, the cells were gently resuspended and the number of spontaneous rosette-forming lymphocytes enumerated. Cells were counted as rosette-positive if three or more SRBC had adhered to them.

Separation of E-binding lymphocytes. E rosettes were prepared as described above. The cell mixture was carefully layered onto a F.I. gradient using an equivalent of 5×10^6 lymphocytes/2 ml gradient and centrifuged at 800 *g* for 20 min at room temperature. The E rosette-forming cells in the pellet and the non rosette-forming cells in the interphase were collected separately and washed three times with culture medium. Each fraction was then treated with 0·83% ammonium chloride solution to lyse erythrocytes and stained for PAM.

EAC-binding lymphocytes. SRBC were washed three times with HBSS. A 5% suspension was sensitized with an equal volume of a 1 in 2000 dilution of rabbit anti-SRBC Serum for 1 hr at 37°C. Sensitized cells were then washed twice and incubated for 30 min at 37°C with fresh human serum, diluted 1 in 10 with HBSS, as a source of complement. After two washes 0·25 ml coated SRBC ($1·5\times 10^8$/ml) was mixed with 0·25 ml of lymphocyte suspension (4×10^6/ml) in Medium 199. The cells were centrifuged (150 *g*, 5 min) and then incubated for 30 min at 37°C. Resuspension of the cells was achieved with a 'whirlmixer' and the percentage of lymphocytes binding three or more SRBC determined.

Simultaneous determinations. Lymphocytes were stained for PAM with fluorescein-conjugated antiserum, as previously described. After washing, the cells were rosetted with E or EAC, or stained for surface immunoglobulin with rhodamine-labelled goat antiserum. Each of these simultaneous determinations was also carried out in the reverse order to ascertain whether the presence of anti-PAM interfered with the second procedure.

Macrophage-migration inhibition test (*George & Vaughan*, 1963). Peritoneal exudate cells were harvested from guinea-pigs 3 days after i.p. injection of 8 ml brain–heart infusion mixture (Difco). The macrophages were washed twice in Medium 199 (4°C), containing antibiotics and 20% v/v heat-inactivated foetal calf serum, and mixed with leucocytes from Mantoux-positive subjects (fractionated by albumin gradient centrifugation) in a ratio of 25:1. Four 20 μl capillary tubes per test were filled with the cell suspension ($1·5\times 10^6$ cells/tube) and each one plugged with an inert clay. The capillaries were centrifuged (120 *g*, 10 min), cut at the cell-liquid interface and mounted in migration chambers (Sterilin) containing culture medium and 50 μg/ml BCG (Wellcome). After 20 hr incubation (37°C) the migration areas were mapped by projection of the chambers through a photographic enlarger and measured by planimetry.

RESULTS

Peripheral blood leucocytes from late pregnant women were subjected to successive washes to ascertain whether the PAM was only loosely bound to the cells. However, no significant reductions in the percentage of PAM-bearing leucocytes was achieved if the cells were washed more than three times (Table 1).

TABLE 1. Effect of successive washes on the presence of PAM on leucocytes*

No. washes†	PAM-bearing leucocytes (%)‡
0	28·4±5·7
1	20·8±3·9
2	13·5±2·4
3	10·8±2·8
4	11·3±1·4
5	12·9±3·0
6	12·1±1·8
7	12·5±2·2
8	11·4±2·8

* Leucocytes from late pregnant women having PAM serum concentrations > 1350 μg/ml.

† Washes carried out with PBS.

‡ Results expressed as mean values of four determinations (±s.d.). PAM detected by indirect immunofluorescence.

The percentage of PAM-associated leueocytes in the blood of a number of males, contraceptive steroid-treated females and pregnant women is plotted against the corresponding PAM-serum concentration in Fig. 1. The mean values (±s.d.) for the percentage PAM-bearing cells and PAM concentrations

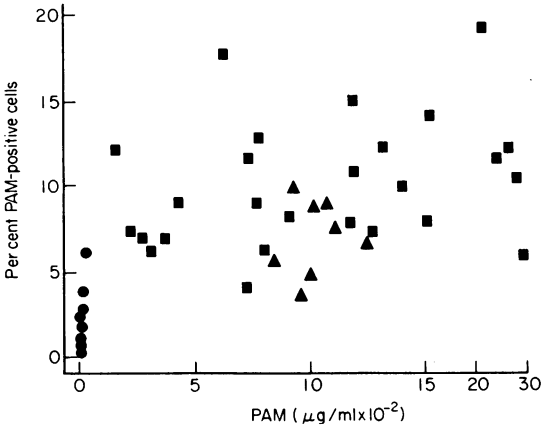

FIG. 1. Relationship between the percentage of peripheral blood leucocytes staining for PAM and the serum-concentration of the glycoprotein. (●) Normal males; (▲) contraceptive steroid-treated females; (■) pregnant women (gestation, 37–40 weeks).

shown in the diagram are as follows: males, 2·8±2·0%, 8·5±8·3 μg/ml; contraceptive steroid treated females, 7·1±2·1%, 1044±129 μg/ml; pregnant women, 9·9±3·8%, 1190±834 μg/ml, respectively. The mean percentages of PAM-bearing cells in pregnant and steroid-treated women were not different ($0{\cdot}1>P>0{\cdot}05$) but both these groups had a significantly higher proportion of PAM-positive cells than the normal males ($P<0{\cdot}001$). However, males with no detectable PAM in their serum had insignificant numbers of PAM-associated leucocytes when compared with those in which the glycoprotein was readily identifiable ($0{\cdot}01>P>0{\cdot}005$). Also, there was no good correlation between PAM concentration and the percentage of PAM-bearing leucocytes: correlation analysis, males $n = 8$, $r = 0{\cdot}695$, $0{\cdot}1>P>0{\cdot}05$; steroid-treated females $n = 8$, $r = 0{\cdot}283$, $P>0{\cdot}1$; pregnant women $n = 26$, $r = 0{\cdot}236$, $P>0{\cdot}1$.

Leucocytes from late pregnant women were subjected to isopyknic density-gradient centrifugation on albumin gradients and the distribution of PAM-associated leucocytes in the six fractions that were collected is indicated in Table 2. Fractions 4–6 were enriched in PAM-positive cells and were found to consist of a high proportion of mononuclear cells. These low-density fractions were also shown to contain the highest percentage of E-rosetting lymphocytes and to be associated with those cells involved in the transfer of delayed hypersensitivity to BCG, as measured by the macrophage migration-inhibition technique. The highest proportion of Ig-bearing lymphocytes was found in Fraction 3, whereas granulocytes were concentrated in Fractions 1 and 2: no enrichment of PAM-associated cells was seen in these fractions.

Fractionation of pregnancy leucocytes on F.I. gradients (Table 3) confirmed the association of PAM with mononuclear cells as the percentage PAM-positive cells was enhanced at the interphase. However, when mononuclear cells were E rosetted and then subjected to a second F.I. separation, PAM-bearing cells were found both at the interphase and in the pellet; similar treatment of lymphocytes resulted in virtually all of the PAM-associated fluorescence being found in the T-cell enriched pellet.

The results of the simultaneous detection of PAM and three surface markers on lymphocytes are shown in Table 4. There was no correlation between cells bearing Ig or forming EAC-rosettes and those associated with PAM. However, it was apparent that an average of 22·9% of E-rosetting lymphocytes also carried PAM on their cell membranes. Simultaneous detections carried out in the reverse order (direct immunofluorescent staining for PAM after detection of the marker) had no effect on the proportions of Ig—or EAC-bearing cells. However, 8–13% increases in the total number of E-rosetting lymphocytes suggested that the presence of rabbit-anti PAM Ig did interfere, to a small extent, with the binding of SRBC to T-cells.

Adherent-cell monolayers were prepared from the peripheral blood leucocytes of pregnant women as previously described. These were stained for PAM by the direct and indirect immunofluorescence

TABLE 2. Density gradient centrifugation of leucocytes from pregnant women

Fraction	Density* (g/cm³)	PAM-bearing cells (%)†	Ig-bearing cells (%)‡	E-rosetting cells (%)*‡	Macrophage migration-inhibition (%)*§	Differential cell count*			Total cell count (× 10⁶)*
						Granulocytes	Lymphocytes	Monocytes	
1	1·094	1·8	2·4	0·4	106	95	5	0	39·6
2	1·085	4·8	9·3	8·1	103	76	22	2	3·7
3	1·075	2·6	23·9	33·7	91	10	86	4	3·4
4	1·067	26·4	5·1	69·4	54	3	89	8	6·3
5	1·060	26·6	5·8	73·3	44	5	77	18	10·2
6	1·052	16·1	8·2	51·1	53	11	74	15	6·1
Total¶	—	11·7	6·0	23·8	63	62	30	8	82·6

* Results expressed as mean values of three determinations.
† Results expressed as mean values of five determinations using indirect immunofluorescence.
‡ Calculated from separations carried out with mononuclear cells obtained after centrifugation with Ficoll–Isopaque.
§ Macrophage migration area expressed as a percentage of migration in the absence of BCG.
¶ Total leucocytes loaded on albumin density gradient.

TABLE 3. Distribution of PAM-bearing cells* on Ficoll–Isopaque gradients

	PAM-bearing cells (%)†		
	Total‡	Interphase	Pellet
Ficoll–Isopaque separation	11·3±2·5	20·9±3·3	0·7±0·3§
E-rosette fractionation¶			
Mononuclear cells	22·2±2·9	19·6±3·3	18·2±3·1
Lymphocytes	18·6±4·2	3·1±1·9	21·3±2·5

* Cells from late pregnant women having PAM serum levels > 1350 μg/ml.

† Results expressed as mean values of three determinations (±s.d.). PAM detected by indirect immunofluorescence.

‡ Values obtained before gradient separation of cells.

§ Cells consisted of > 98% granulocytes.

¶ Mononuclear and non-adherent cells, obtained after a preliminary FI separation, were subjected to further fractionation by the rosette sedimentation technique.

TABLE 4. Simultaneous detection of PAM and surface markers on lymphocytes* obtained from pregnant women

Surface marker	Cells bearing marker alone (%)†	Cells bearing PAM alone (%)†‡	Cells bearing both PAM and marker (%)†‡
Immunoglobulin	19·3±5·2	23·1±5·5	0·6±0·2
EAC-rosettes	24·9±4·8	19·6±3·4	1·1±0·9
E rosettes	52·9±6·1	2·2±1·3	15·7±3·6

* Cells employed were shown to consist of > 98% lymphocytes.

† Results expressed as mean values of four determinations (±s.d.).

‡ PAM detected by direct immunofluorescence.

procedures and it was found (Table 5) that in both cases approximately 40% of the monocytes carried the glycoprotein on their cell membranes.

The membrane immunofluorescence observed on leucocytes with fluorescein-conjugated anti-PAM was completely inhibited by treating the cells with unlabelled anti-PAM but was unaffected by pre-treatment with NRS or soluble antigen–antibody complexes. Anti-PAM which had been completely absorbed with the glycoprotein no longer retained its ability to stain cells by direct immunofluorescence, however use of the $F(ab')_2$ fragment did result in normal staining. All indirect determinations were carried out with NRS and anti-albumin controls. Also, each antiserum employed was subjected to high-speed centrifugation to remove any aggregates and contained sodium azide, whenever feasible. It is

TABLE 5. Detection of PAM on peripheral blood monocytes from pregnant women

Type of immunofluorescent staining	PAM bearing adherent cells (%)*†
Indirect	41·5±7·3
Direct	38·1±5·2

* Adherent cells employed were shown to consist of > 95% monocytes.

† Results expressed as mean values of nine separate determinations (±s.d.).

therefore probable that the immunofluorescence noted is due to specific interaction of anti-PAM with membrane-associated PAM rather than to non-specific uptake, perhaps involving phagocytosis, or Fc or C3 receptors on cells. The majority of specimens exhibited a uniform granular fluorescence but, in the absence of azide, distinct capping was also observed in the lymphocyte preparations.

DISCUSSION

Pregnancy-associated α-macroglobulin was identified on the surface of peripheral blood leucocytes. The percentage of PAM-positive cells was significantly higher in subjects with elevated PAM-serum levels (pregnant and contraceptive-steroid-treated women) than in those with low concentrations of the α-globulin (normal males). However, no correlation was found between PAM concentrations and the number of leucocytes exhibiting membrane-associated PAM, indicating that the presence of PAM on these cells was not simply due to non-specific adsorption. This suggestion is supported by the fact that the glycoprotein could not readily be removed from all positive leucocytes by washing. Also, subjects with no detectable serum-PAM had insignificant numbers of PAM-associated cells and therefore it is most unlikely that the antigen detected on the leucocytes was a normal constituent of the cell membrane. It is possible that only a limited number of cells have the ability to bear PAM and that above a relatively low blood-concentration the binding sites on this sub-population are saturated with glycoprotein. This suggestion is supported by investigations into the immunosuppressive properties of PAM. The glycoprotein was shown capable of causing significant partial reductions in lymphocyte reactivity *in vitro*. Maximum inhibition was achieved at approximately 400 μg/ml, a value well below that normally found in late pregnancy, and no further alteration in lymphocyte response was observed at concentrations above this figure (Stimson, 1976).

Separation of leucocytes from pregnant women by density-gradient centrifugation indicated that PAM was particularly associated with relatively low-density mononuclear cells, which formed E rosettes and were involved in the transfer of delayed hypersensitivity but did not bear Ig on their surface. Granulocyte-rich fractions were not enriched with PAM-positive cells and fractionation of pregnancy leucocytes on F.I. gradients confirmed that this cell type was not associated with the glycoprotein. When lymphocytes were subjected to the simultaneous detection of PAM and three surface markers it was found that approximately 23% E-rosetting cells carried PAM, whereas the numbers of EAC and Ig-associated lymphocytes bearing the α-globulin were relatively insignificant. These results indicate that PAM has the ability to bind to lymphocytes but that this capacity is limited to a proportion, possibly a sub-population, of T lymphocytes. Further evidence for such an association with T lymphocytes comes from the observation that PAM can significantly suppress lymphocyte transformation induced by agents often regarded as preferential stimulators of T cells but that it was much less effective in cultures in which B cells were selectively stimulated. (Stimson, 1976). Another non-immunoglobulin serum protein has been reported to be present on the surface of human lymphocytes. James *et al.* (1975) found that α_2-macroglobulin was associated with B-cells in both normal subjects and patients with chronic lymphocytic leukaemia. Antiserum to this glycoprotein inhibited K-cell activity but had no effect on T (E) or B (EAC) rosette formation (Calder *et al.*, 1975). In the present study, pretreatment of lymphoid cells with anti-PAM depressed their capacity to form T rosettes but did not significantly reduce EAC rosetting, indicating that the two α-macroglobulins are associated with different types of lymphocyte.

Centrifugation of pregnancy leucocytes on albumin gradients also showed that the fractions containing the majority of PAM-positive cells were enriched in monocytes. The association of PAM with monocytes was further suggested by the finding that cells bearing the glycoprotein could only be removed from the interphase of F.I. gradients by the T-cell-rosette removal technique when the cells employed were pure lymphocytes: use of mononuclear cells in the technique resulted in a proportion of PAM-positive cells remaining at the interphase. Confirmation of this association was obtained when PAM was detected on approximately 40% adherent cells by both direct and indirect fluorescent procedures.

This observation may be indicative of distinct monocyte subpopulations; in the tissues the 'mature' form of this cell type, the macrophage, is recognised as exhibiting heterogeneity (Cline, 1975). However, it is equally possible that they represent monocytes at different stages of development.

The role of PAM on the surface of monocytes is uncertain but an investigation (to be published), in which these cells were found to possess the capacity to synthesise the glycoprotein, suggests one explanation for the phenomenon. Studies have shown that PAM-blood concentrations can be elevated in cancer patients and that variations in the level of the glycoprotein in subjects receiving treatment correlate with the course of the disease, often showing a significant 'lead-time' over clinical diagnosis (Stimson, 1975a). The association of PAM with mononuclear phagocytes could be relevant to these observations as it has been reported (Evans, 1972; Eccles & Alexander, 1974) that tumours can contain large numbers of cells of the monocyte/macrophage series.

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