Hormone-Induced Cell Death

2. Surface Changes in Thymocytes Undergoing Apoptosis

R. G. MORRIS, MSc, A. D. HARGREAVES, BSc, MBChB, E. DUVALL, D Phil, MBChB, and A. H. WYLLIE, PhD, MRCPath

From the Department of Pathology, University of Edinburgh Medical School, Edinburgh, Scotland

***In vivo*, apoptotic cells are swiftly recognized by phagocytes, presumably because of changes on their surface. This article describes surface changes in rat cortical thymocytes undergoing apoptosis induced by glucocorticoid treatment *in vitro*. Homogeneous populations of thymocytes early in apoptosis were prepared by isopyknic centrifugation. These cells were compared with purified nonapoptotic cells in terms of several surface characteristics, including binding to macrophages, surface ultrastructure, microelectrophoretic mobility (a measure of surface charge density), and ability to bind four lectins and four monoclonal antibodies to thymocyte antigens. Apoptotic cells bound to macrophages more avidly than did nonapoptotic cells by a process not dependent upon serum factors. Their surfaces lost microvilli and became "blistered," apparently through fusion of vesicles of endoplasmic reticulum with the plasma membrane. The surface charge density of apoptotic cells was less than that of nonapoptotic cells. Surface antigens and lectin-binding sites were less abundant on apoptotic than on normal cells, in proportion to the general reduction in cell size observed in apoptosis. Differences between apoptotic and normal cells were not detected, however, in the relative quantities of exposed galactose, N-acetyl galactosamine, N-acetyl glucosamine, N-acetyl neuraminic acid, or of several surface antigens, including the major sialoglycoproteins of the thymocyte membrane. It appears that although several changes occur in the surface of apoptotic cells, many cell membrane structures remain intact. The changes responsible for the recognition of apoptotic cells by phagocytes are more subtle than those detectable by the binding of lectin and antibody probes, but preliminary data suggest that a lectin-sugar interaction is involved. (Am J Pathol 1984, 115:426–436)**

APOPTOSIS is a process whereby nucleated cells are deleted from living tissues.[1,2] It occurs as the "programmed death" of cells in developing tissues, in normal tissue turnover, in hormone-induced atrophy, in metamorphosis, and in the growth and regression of tumors. Ultrastructural studies suggest that in tissues, the dying cells are recognized and phagocytosed by their neighbors soon after initiation of apoptosis,[2,3] which appears to be an abrupt process.[4] In tissues with exceptionally high rates of cell turnover, phagocytes filled with apoptotic cells are conspicuous histologic features (for example, the "tingible body" macrophages of reactive lymphoid tissue[5]); but even where turnover is slower, recognition and phagocytosis of apoptotic cells may play a role in tissue organization. The process is capable of removing potentially toxic cellular materials from the intercellular space and facilitates the recycling of certain molecules in the tissue. Little is known, however, of the mechanisms whereby apoptotic cells are recognized. Although it is reasonable to suppose that alterations occur on the surface of apoptotic cells which permit phagocyte binding, this has not been demonstrated hitherto. To our knowledge, the aging erythrocyte is the only dying cell whose recognition by macrophages has been systematically studied.[6] Evidence suggests that surface changes, together with serum factors, including complement[7] and antibody,[8] may play roles in erythrocyte phagocytosis, but the erythrocyte is atypical in many ways and may provide a misleading model for the recognition of apoptotic nucleated cells. In this article, the surface of apoptotic rat thymocytes is evaluated by several criteria. Thymocytes are particularly suited to studies on apoptosis, be-

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Address reprint requests to Dr. A. H. Wyllie, Department of Pathology, University Medical School, Teviot Place, Edinburgh UK EH8 9AG.

cause the process can be induced by a short exposure to glucocorticoid hormones *in vitro*; further, apoptotic cells can be procured in nearly homogeneous suspensions[4] and compared with homogeneous populations of morphologically normal cells. We demonstrate that there are surface changes in thymocytes undergoing apoptosis which are capable of affording recognition signals to macrophages; we show that these changes are highly selective, because several marker molecules present on the normal thymocyte surface are apparently unchanged; and we present a hypothesis for the means whereby these surface changes arise, based upon ultrastructural and biophysical features.

Materials and Methods

Thymocyte Suspensions

Using methods described in detail elsewhere,[9] thymus glands from suckling Sprague–Dawley rats were chopped in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated newborn calf serum to provide suspensions of cortical thymocytes. The cell suspensions were incubated with or without glucocorticoid (10^{-5} M methylprednisolone or 10^{-7} M dexamethasone) for 4–6 hours and then subjected to isopyknic centrifugation on step or continuous Percoll gradients for separation of the high-density apoptotic cells from morphologically normal cells with low density as previously described.[4] Percoll was washed from the cells prior to further study, by dilution in MEM and centrifugation (200*g* for 10 minutes at 4 C).

Binding Assays

Monolayers of peritoneal macrophages were prepared from 1–2-month-old outbred Schofield mice as previously described.[10] Suspensions of 10^6 peritoneal cells in 1 ml MEM, supplemented with 25 mM morpholinopropane sulfonic acid (MOPS) pH 7.4, 5 IU heparin, and 5% heat-inactivated pooled rat serum, were placed in siliconized glass tubes, each containing a nonsiliconized flying coverslip. After 18 hours of incubation at 37 C in a humidified atmosphere of 5% CO_2 in air, to permit cell attachment, the monolayers were washed twice in MEM buffered with 25 mM MOPS, pH 7.4 (MEM-MOPS), and incubated for a further hour in this medium alone, in an attempt to remove heparin and serum prior to the phagocytosis assay. For the assay, the overlying medium was replaced by 1 ml MEM-MOPS containing 2×10^6 thymocytes. In experiments designed for study of the influence of serum factors on phagocytosis, 5% normal or heat-inactivated rat serum was added at this stage. All tubes were then reincubated without agitation for 100 minutes at 37 C. The coverslips were washed twice in MEM-MOPS, fixed in 95% methanol, and stained by the Giemsa method. On each coverslip, 500 macrophages were counted. Thymocyte nuclei, which were readily recognizable on the basis of their size, were scored as bound if their profile transected the outline of the macrophage. In this assay no distinction was made between ingestion and binding, although it was clear morphologically that only a proportion of the bound cells lay within phagosomes. Macrophages were scored as positive if they had bound 1 or more thymocytes. Certain monolayers in all experiments received no additional cells or 5×10^7 ox erythrocytes, which were coated with either rabbit IgG or human complement.[11] Only if they *ingested* one or more erythrocytes were macrophages scored as positive in these control coverslips which were intended to confirm the phagocytic potential of the macrophages but exclude the possibility that they had been "activated" to ingest particles nonspecifically.[12]

In a small series of experiments with macrophage monolayers this protocol was modified to test the effect of monosaccharides on the macrophage–thymocyte binding. After preparation of the monolayers by overnight incubation as before, they were washed twice in complete Dulbecco's phosphate-buffered saline (containing 1 mM Ca and 0.5 mM Mg) (DPBS) and incubated in this medium for a further 60 minutes at 37 C. Thereafter, the temperature was lowered to 4 C, and the medium was replaced with fresh DPBS, with or without supplementation with 20 mM N-acetyl D-glucosamine or D-mannose. After a further 15 minutes, the medium was replaced with a suspension of apoptotic thymocytes, prepared as before, but in DPBS supplemented with the same monosaccharides. The monolayers were prepared for scoring as before after 120 minutes' incubation at 4 C.

In a further series of experiments thymocyte–macrophage binding was assessed in suspension. In conical centrifuge tubes 2×10^5 peritoneal cells were mixed with 10^7 thymocytes in a total of 200 μl MEM-MOPS. The suspensions were centrifuged at 45*g* for 10 minutes at 4 C; 100 μl of the supernatant was removed, and the pellet was gently resuspended by tapping. After incubation at 4 C for a further 40 minutes, the cell suspension was assessed for the percentage of rosetting macrophages, by fluorescence microscopy of wet preparations stained with acridine orange. A rosette was defined as a macrophage with

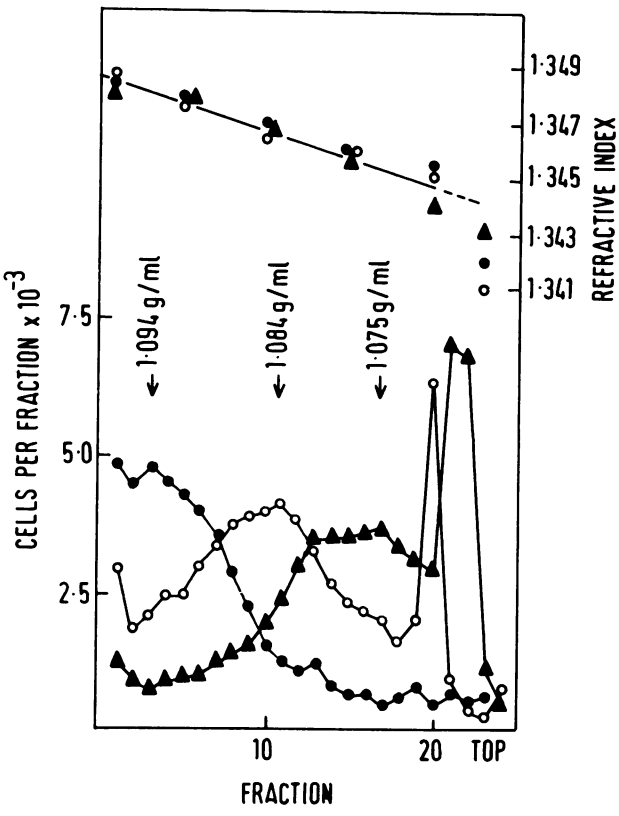


Figure 1 — Density profiles of apoptotic thymocytes incubated at 37 C. The curves show the cell numbers in consecutive fractions of 50–100% continuous Percoll gradients, loaded with apoptotic thymocytes originally obtained from the 80–100% interface of a discontinuous Percoll gradient following 4-hour exposure to 10^{-5} M MP *in vitro*. Cells were applied to the continuous gradient after 0-hour (●), 2-hour (O) and 4-hour (▲) incubations in culture medium at 37 C. Also shown are the refractive index profiles of the three gradients.

3 or more attached thymocytes. Two hundred macrophages were counted from each tube.

Cell Microelectrophoresis

Following the method of Van Oss et al,[13] thymocytes were washed in low ionic strength buffer (11 mM Na_2PO_4, 0.36 mM citric acid, and 270 mM glucose) and resuspended in the same buffer. They were electrophoresed in 75-mm capillary tubes (MSE Ltd.) coated with 2.5% agarose (Miles Laboratories Ltd.) in a field of 10 V/cm. The distance traveled in 30 seconds was measured with the use of an eyepiece graticule and phase-contrast optics, the polarity was reversed, and the distance traveled in 30 seconds in the opposite direction was also measured. The mean of these two observations was taken for 30 cells in each experiment.

Neuraminidase Digestion

Thymocytes were washed twice in phosphate-buffered saline, pH 7.2 (PBS, Oxoid Ltd.), resuspended in PBS containing 2 units/ml of neuraminidase (Type VI, Sigma Chemical Co.) and incubated for 1 hour at 37 C.[14] Control cells were incubated in PBS without enzyme.

Binding of Fluoresceinated Lectins

Thymocytes were washed twice either in PBS or (for *Limulus polyphemus* binding) in 0.05 M Tris, pH 7.5, 0.01 M $CaCl_2$, 0.15 M NaCl, and resuspended with various lectins conjugated to fluorescein isothiocyanate (FITC) (Tissue Culture Services Ltd.) to give 10^6 cells and 100 μg lectin/ml. Incubation was for 30 minutes at room temperature. Inhibitory sugars, when present, were at a final concentration of 0.2 M. The cells were washed three times after incubation and analyzed in a Beckton-Dickinson FACS IV cell sorter. A plot was obtained of the distribution of total fluorescence per thymocyte after lectin binding both in the absence and in the presence of the sugar for which the lectin was specific. The modal value of fluorescence for each population was chosen as a single index allowing comparison between populations.

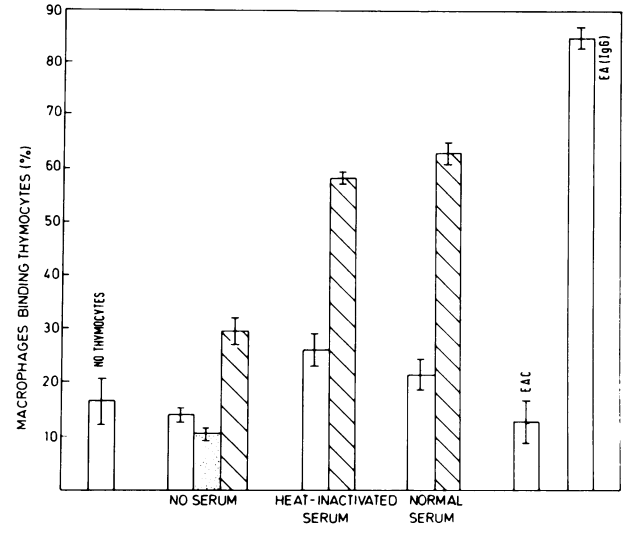


Figure 2 — Thymocyte binding to macrophage monolayers at 37 C. The percentage of macrophages (± 1 SE) binding of the applied cells is shown. Thymocytes were harvested from discontinuous Percoll gradients after 6 hours of treatment with 10^{-7} M dexamethasone. The *hatched bars* show binding of apoptotic thymocytes; the *stippled bar* indicates morphologically normal, steroid-treated thymocytes, and the *open bars* represent untreated thymocytes. In the absence of serum during incubation with the macrophages, the binding of morphologically normal cells was indistinguishable from background levels where no thymocytes were applied to the monolayers ("NO THYMOCYTES"). The results of incubation in the presence of 5% pooled rat serum, with and without heat inactivation (56 C for 40 minutes) are also shown. Over 80% of the macrophages in these monolayers were able to ingest ox red cells opsonised with IgG (EA [IgG]), but less than 20% ingested red cells opsonised with complement (EAC).

Table 1—Thymocyte Binding to Macrophages in Suspension at 4 C

Thymocytes Density	Dex*	No. of experiments	Rosetting macrophages (mean % ± 1 SE)
low	–	18	19.8 ± 2.8
low	+	21	18.1 ± 1.9†
high	+	24	45.0 ± 3.4†

* In this experiment steroid treatment was with 10^{-7} M dexamethasone for 6 hours.
† $P < 0.001$ (Student t test).

Binding of Monoclonal Antibodies

Culture supernatants from hybridomas producing monoclonal antibodies against a variety of rat leukocyte surface markers were kindly supplied by Dr. A. F. Williams (MRC Cellular Immunology Unit, Oxford). Rat thymocytes were prepared as for lectin binding, but 3×10^6 cells were incubated in culture supernatant for 30 minutes, washed twice by centrifugation in PBS, and then incubated with a FITC-conjugated goat anti-mouse immunoglobulin serum (Mercia-Brocades, Ltd.) at 1:5 dilution for half an hour. Negative controls were incubated in goat anti-mouse immunoglobulin alone. The cells were then washed twice in PBS, fixed in 1% formaldehyde in PBS for 30 minutes, and examined by flow microfluorimetry as above.

Sizing of Thymocytes

Rat thymocytes were sized on a Coulter Counter Model ZB1 using a size distribution analyzer P128 with a 100-μ aperture, aperture current of 1/4, and an amplification of 2. Thymocytes were diluted in Isoton II (Coulter Electronics Ltd.), and 10,000 cells were analyzed. Calibration of the system was performed with latex beads of known size.

Ultrastructural Methods

Lectin binding was visualized by electron microscopy using colloidal gold. Thymocytes were prefixed in 1% glutaraldehyde in 0.075 M sodium cacodylate buffer and stained with gold-conjugated lectins. Colloidal gold was prepared and conjugated to wheat-germ agglutinin (Calbiochem Ltd.)[15] or *Ricinus communis* agglutinin I (Sigma).[16] For visualization of colloidal iron binding, similarly fixed cells were washed twice in 3% glacial acetic acid and stained for 2 hours in 1% colloidal iron (BDH Ltd.) in 3% acetic acid.[17] Excess stain was washed out in the acetic acid solution, and the cells were processed for transmission electron microscopy. Ultrastructural localization of acid phosphatase was studied by a modified Gomori method,[18] using cells fixed for 10 minutes in 2% glutaraldehyde and incubated in a combination of α- and β-glycerophosphate (each at 1 mg/ml) and lead citrate (1 mg/ml) in 7.5% sucrose, 50 mM sodium acetate buffer, at the optimum pH of 5.5. Incubation was for 30 minutes at 37 C with shaking. Thereafter, the cells were washed in substrate-free 7.5% sucrose, 50 mM acetate buffer, and postfixed in 1% osmium tetroxide and 0.1 M sodium cacodylate buffer, pH 7.4, prior to processing for microscopy. Thin sections were viewed in a JEOL 100S transmission electron microscope.

Results

Selection of Apoptotic Cells by Isopyknic Centrifugation

The apoptotic cells in this study were separated from their morphologically normal neighbors on the basis of increased density. We showed previously[4] that the buoyant density of glucocorticoid-treated thymocytes rises in a single abrupt step, coincident with the appearance of the morphologic features of apoptosis, at the earliest around 1 hour after commencing exposure to steroid. In the present experiments apoptotic cells were shown to lose buoyant density progressively and rapidly on further incubation (Figure 1). Data from this figure showed that the proportion of cells with density in excess of 1.086 g/ml decayed exponentially over 4 hours of incubation, with a half-life of 1.6 hour. This implies that the majority of apoptotic cells, harvested on the basis of density >1.086 after 4–6 hours of incubation with steroid, had entered the phase of increased density less than 3 hours previously.

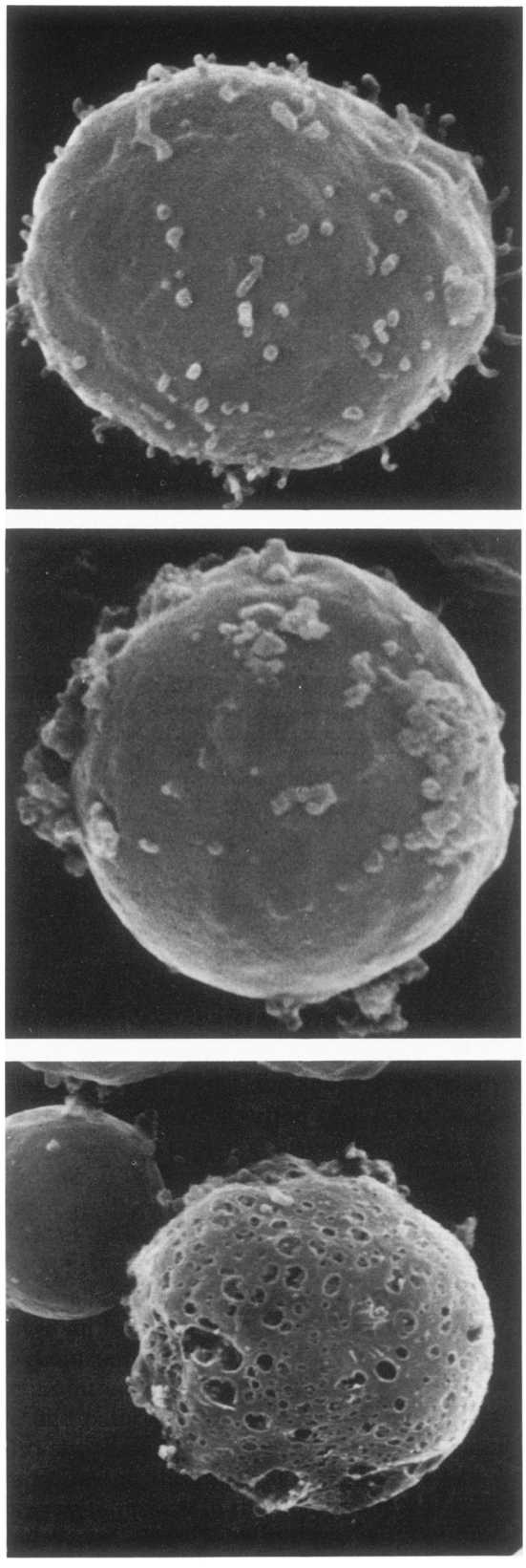
Preferential Binding of Apoptotic Cells to Macrophages

On incubation with mouse peritoneal macrophages, apoptotic cells were bound more readily than

Table 2—Inhibition of Binding of Apoptotic Thymocytes to Macrophages by Monosaccharides

Monosaccharide (20 mM)	Macrophages binding thymocytes (mean % ± 1 SE)
Nil	15.9 ± 3.0
D-mannose	17.4 ± 1.6
N-acetyl-D-glucosamine	4.4 ± 1.3

The values given are the means of three experiments. Binding of apoptotic cells in the presence and absence of N-acetyl-D-glucosamine differs at the significance level $P = 0.05$ (one-tailed Mann-Whitney U test). The differences in absolute levels of binding in the absence of monosaccharide in this experiment, compared with Figure 2, are probably attributable to the different incubation conditions.



thymocytes of normal density and morphology (Figure 2). It is difficult to assign a simple numeric value to the increase in affinity of macrophages for apoptotic cells. Under the conditions of assay about 30% of macrophages bound to apoptotic thymocytes in the absence of serum, whereas 15% bound to morphologically normal cells, whether or not these had been exposed to steroid. However, macrophage monolayers incubated in the complete absence of thymocytes also contained about 15% cells with attached or intracytoplasmic chromatin particles—presumably the remains of cells originally present in the peritoneal washings but dying during overnight incubation of the monolayers. Hence, there is no evidence that, in the absence of serum, morphologically normal thymocytes were bound by macrophages at all. In the presence of serum, binding of both apoptotic and normal cells was increased, but in monolayers exposed to apoptotic cells 2–4 times more macrophages bound thymocytes than in those exposed to normal cells. This effect of serum was not due to complement, because it was heat-stable (Figure 2).

In these experiments the macrophages were shown to be in a "nonactivated" state, as revealed by their inability to *ingest* complement-coated erythrocytes in parallel cultures,[12] although, as expected of cells bearing C3 receptors, more than 90% rosetted with these particles. Phagocytic potential was confirmed by the ability of more than 80% of macrophages to ingest IgG-coated erythrocytes. Additional experiments showed that macrophages were not "activated" to ingest complement-coated erythrocytes by incubation in medium conditioned by apoptotic thymocytes.

The increased binding of apoptotic cells by macrophage monolayers was shown not to be attributable merely to more rapid sedimentation of the apoptotic cells onto the monolayers. The rate of sedimentation of apoptotic cells through culture medium was measured directly and did not differ significantly from the rate of sedimentation of morphologically normal cells. Further, when thymocytes were mixed with peritoneal macrophages in suspension at 4 C and cosedimented by centrifugation, more macrophages formed thymocyte rosettes when the thymocytes were

Figure 3—Scanning electron microscopy of thymocytes. Control, untreated cells (**A**) have microvillous surfaces, whereas the surfaces of apoptotic, high-density cells lack microvilli but show occasional irregular cytoplasmic blebs (**B**) or extensive pitting (**C**). (**A** and **B**, × 13,600; **C**, × 8000)

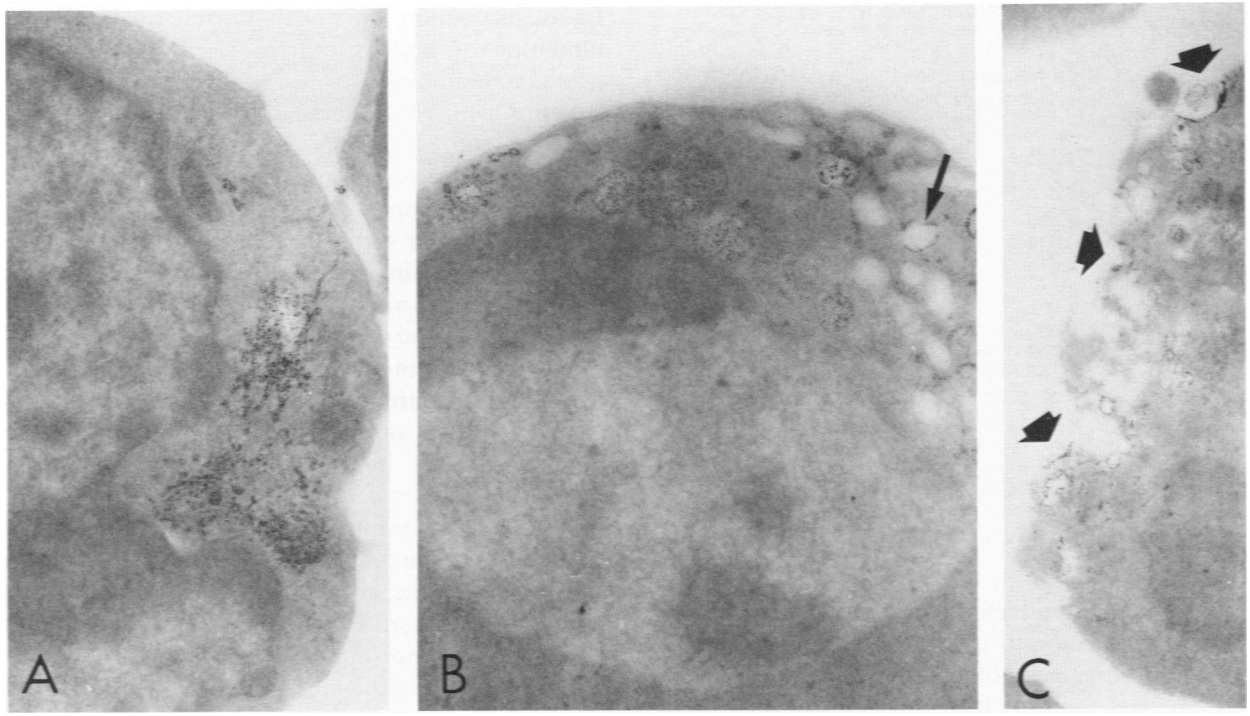


Figure 4 — Acid phosphatase histochemistry of thymocytes. In morphologically normal cells (**A**) the reaction product is located predominantly in the Golgi complex and endoplasmic reticulum (ER) membranes. Some product is also seen within oval organelles of uncertain identity, but the cell surface is unstained. In apoptotic cells, reaction product is seen within cytoplasmic vesicles, which appear to be dilated portions of Golgi complexes or ER (**B**, *arrow*). In some apoptotic cells the product is found lining surface pits (**C**, *broad arrows*). (Unstained sections, × 20,000)

apoptotic than when they were morphologically normal (Table 1).

Finally, the specificity of macrophage binding to apoptotic cells was further tested by examining its reversibility in the presence of various monosaccharides. Whereas D-mannose did not inhibit the binding of apoptotic cells at 4 C, binding was inhibited by N-acetyl D-glucosamine (Table 2).

Morphologic Changes on the Surface of Apoptotic Cells

By scanning electron microscopy apoptotic cells were shown to have lost the microvilli characteristic of normal cortical thymocytes (Figures 3A and B). Small irregular blebs of cytoplasm projected from the surface of the apoptotic cells, perhaps corresponding to the budding fragments of cytoplasm observed with transmission electron microscopy. Some cells also showed a deeply pitted surface (Figure 3C). Acid phosphatase histochemistry applied to transmission electron-microscopic sections showed that the reaction product was confined to endoplasmic reticulum and Golgi profiles in normal cells, but in apoptotic cells it lined dilated cytoplasmic vesicles and also appeared on indented portions of the surface membrane (Figure 4). This suggested that the pits observed in scanning electron microscopy represented accelerated fusion with the surface membrane of membranes from endoplasmic reticulum.

Surface Charge of Apoptotic Cells

Cell microelectrophoresis demonstrated reduced migration of apoptotic cells relative to cells of normal density and morphology (Table 3). Migration was greatly reduced by previous treatment of the cells with neuraminidase, confirming that most of the migration was attributable to negative charge conferred by terminal N-acetyl neuraminic acid (NANA) groups on the cell membrane. Neuraminidase treatment abolished the difference in electrophoretic mobility between apoptotic and morphologically normal cells.

The distribution of charged groups on the cell surface was studied by electron microscopy after exposure to colloidal iron. Apoptotic cells and mor-

Table 3—Thymocyte Electrophoretic Mobilities

Cell density	Neuraminidase digestion	Mean mobility (μ volt^{-1} cm sec^{-1})
Low	–	1.18 (1.14–1.21)*
High	–	1.05 (1.03–1.06)
Low	+	0.46 (0.44–0.48)
High	+	0.49 (0.48–0.51)

Each mobility value is the mean of the medians of two experiments in each of which 30 cell mobilities were assessed. The mobilities for low- and high-density cells, untreated by neuraminidase, differ significantly ($P < 0.0005$; Wilcoxon rank sum test). The reduction in mobility of both high- and low-density cells, after neuraminidase treatment, is also significant ($P < 0.0005$). In these experiments both low- and high-density cells had been exposed to methylprednisolone 10^{-5} M for 4 hours.
* Range.

phologically normal cells bound colloidal iron over their entire surfaces (Figures 5A–C). No apoptotic cells were observed which failed to bind colloidal iron, nor was there evidence of defective binding to patches of membrane of apoptotic or normal cells. In confirmation that the bound iron related to the presence of terminal NANA residues, prior treatment with neuraminidase abolished binding in both apoptotic and morphologically normal cells (Figure 5D).

Binding of Lectins and Monoclonal Antibodies to Apoptotic Cells

We were unable to detect differences in the patterns of binding of gold-labeled *Ricinus communis* agglutinin (RCA I) or wheatgerm agglutinin (WGA) to the surface of apoptotic and morphologically normal cells. In particular, no alterations were observed in the plasma membrane related to the surface blisters in apoptotic cells (Figure 6). Lectin binding was strongly inhibited by coincubation with galactose (for RCA I) or N-acetyl-glucosamine (for WGA).

In an attempt to demonstrate quantitative differences in cell surface sugars, we analyzed cells which had been exposed to FITC-conjugated lectins by flow microfluorimetry. This showed that the modal fluorescence following binding of each of the four lectins tested (RCA I, WGA, LPA, and PNA) was approximately 50% less in apoptotic than in morphologically normal cells (Figure 7). Specificity of binding was established by inhibition in the presence of the appropriate monosaccharides or—in the case of LPA and WGA—by prior digestion of the cells with neuraminidase. In common with other workers,[19] we found incubation with NANA itself unsuitable for inhibition of binding of these lectins. NANA appeared to bind directly to some of the cells, and coincubation with NANA actually increased the binding of WGA and LPA to apoptotic cells.

Seven monoclonal antibodies to rat leukocyte antigens were applied to the cells and traced with FITC-conjugated second antibody. Background fluorescence only, similar to that obtained with second antibody alone, was observed with ox-6 and ox-12. This was expected, because these antibodies are specific for Ia antigen and the F_{ab} fragment of immunoglobulins, respectively—epitopes absent from thymocytes. Ox-18, an antibody specific for an epitope on mature, but not immature, thymocytes, also gave background fluorescence only. Both apoptotic and morphologically normal thymocytes bound ox-8 and w3/25 (against immature thymocytes), w3/13 (against thymocyte surface glycoprotein), and ox-1 (against leukocyte common antigen). In each instance, the modal fluorescence intensity of apoptotic cells was about half that of the morphologically normal cells, whether or not these had been exposed to steroid (Figure 7).

The observation that the fluorescence of apoptotic cells was less than that of morphologically normal cells, after binding several different probes, suggested that the apoptotic cell surface contained binding sites of the same type as those on normal cells but in reduced numbers. The simplest explanation for this is reduction in the total membrane surface area in apoptosis, and this was confirmed by Coulter Counter analysis. The average volume of apoptotic cells was 2.48×10^{-7} μl, whereas that of normal was 5.56×10^{-7} μl. For perfectly spherical cells this would represent a reduction in surface area to 58% of its initial value. This corresponds closely to the reduction in fluorescence intensity observed with both monoclonal antibodies and lectins (mean, 50%).

Discussion

Earlier studies on the recognition and phagocytosis of effete cells have been based on heterogeneous cell systems such as aging or damaged erythrocytes,[6,20] have been restricted to a small number of surface characteristics,[21] have investigated recognition processes dependent upon serum factors,[6–8] or have focused entirely on the process of phagocytosis rather than the essential, preceding phase of recognition.[22] In this work, we have studied the surface of homogeneous populations of cells, proven to be at an early

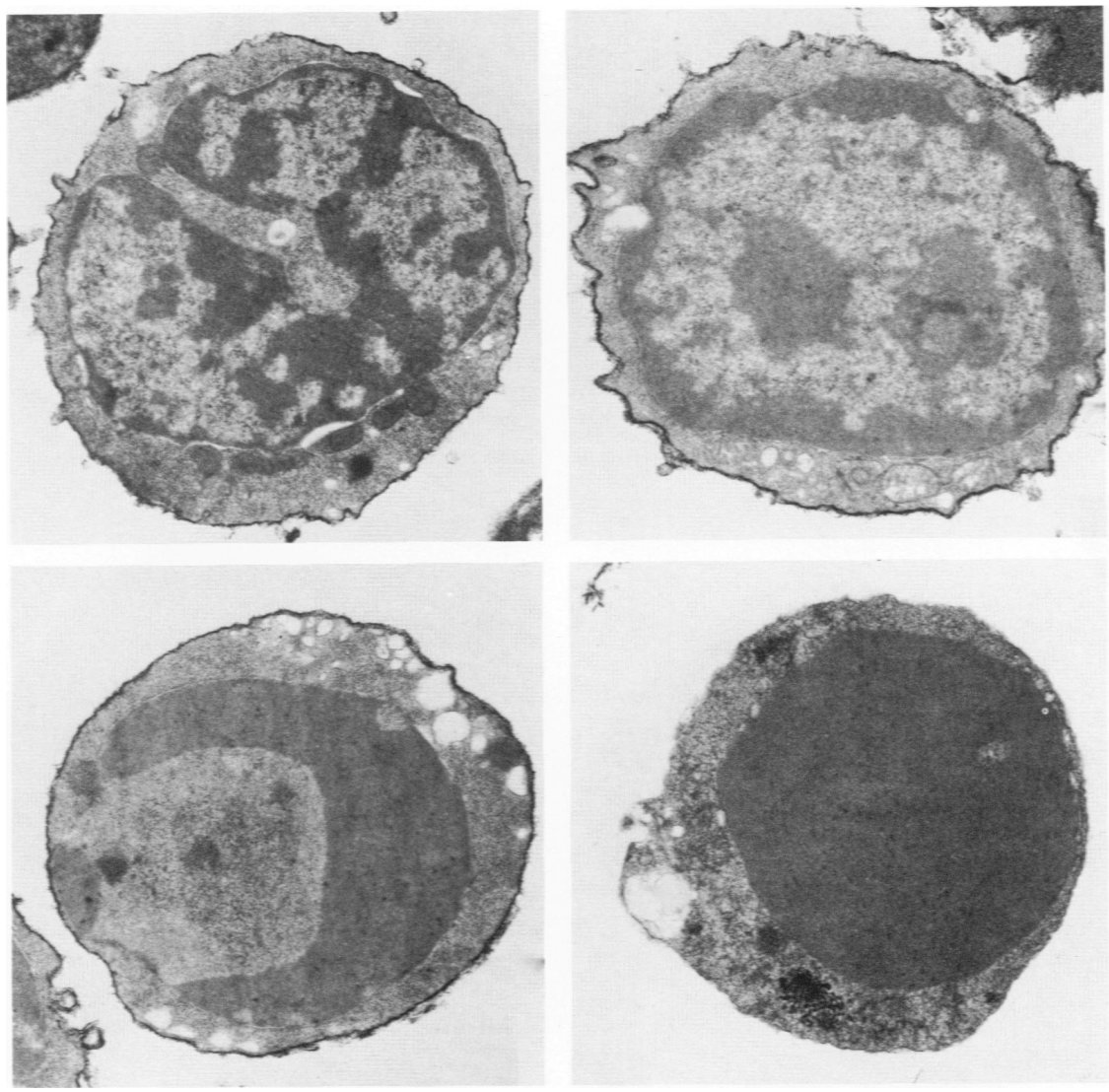


Figure 5 — Colloidal iron binding by thymocytes. The electron-dense layer is continuous around untreated thymocytes (**A**), treated thymocytes of normal density (**B**), and treated, apoptotic cells (**C**). Prior neuraminidase treatment prevented iron binding completely (**D**). Similar results with neuraminidase were obtained with nonapoptotic cells. (Uranyl acetate and lead citrate, **A**, × 13,400; **B**, × 18,000; **C**, × 20,000; **D**, × 22,000)

phase in the process of apoptosis, following a defined hormonal stimulus. This model system is further validated by direct demonstration that macrophages bind selectively to apoptotic thymocytes, without prior "activation," by a process operative at 4 C and in the absence of serum factors.

Although the differences in binding of apoptotic and normal cells are highly significant statistically, it may be argued that they are insufficiently large to account for the efficient phagocytosis of apoptotic cells *in vivo*. The data presented here, however, establish the point that these experiments were designed to test: surfaces of cells undergoing apoptosis are altered within a few hours in such a way as to convey recognition signals to other cells. Clearly there are many differences between the conditions of our assay and those pertaining *in vivo*. Cell contacts are unlikely to be as continuous; in the rosette assay the temperature was 4 C; we do not know whether all peritoneal macrophages are able to recognize apoptotic cells; and the binding assays described refer to a heterologous cell system. In a smaller series of experiments, we observed selective binding of apoptotic rat thymocytes by rat macrophages, but it proved more difficult to obtain the rat macrophages in a "nonactivated" state. We report in more detail elsewhere on the selective binding of mouse apoptotic thymocytes by mouse macrophages.[23] In all these systems binding was ob-

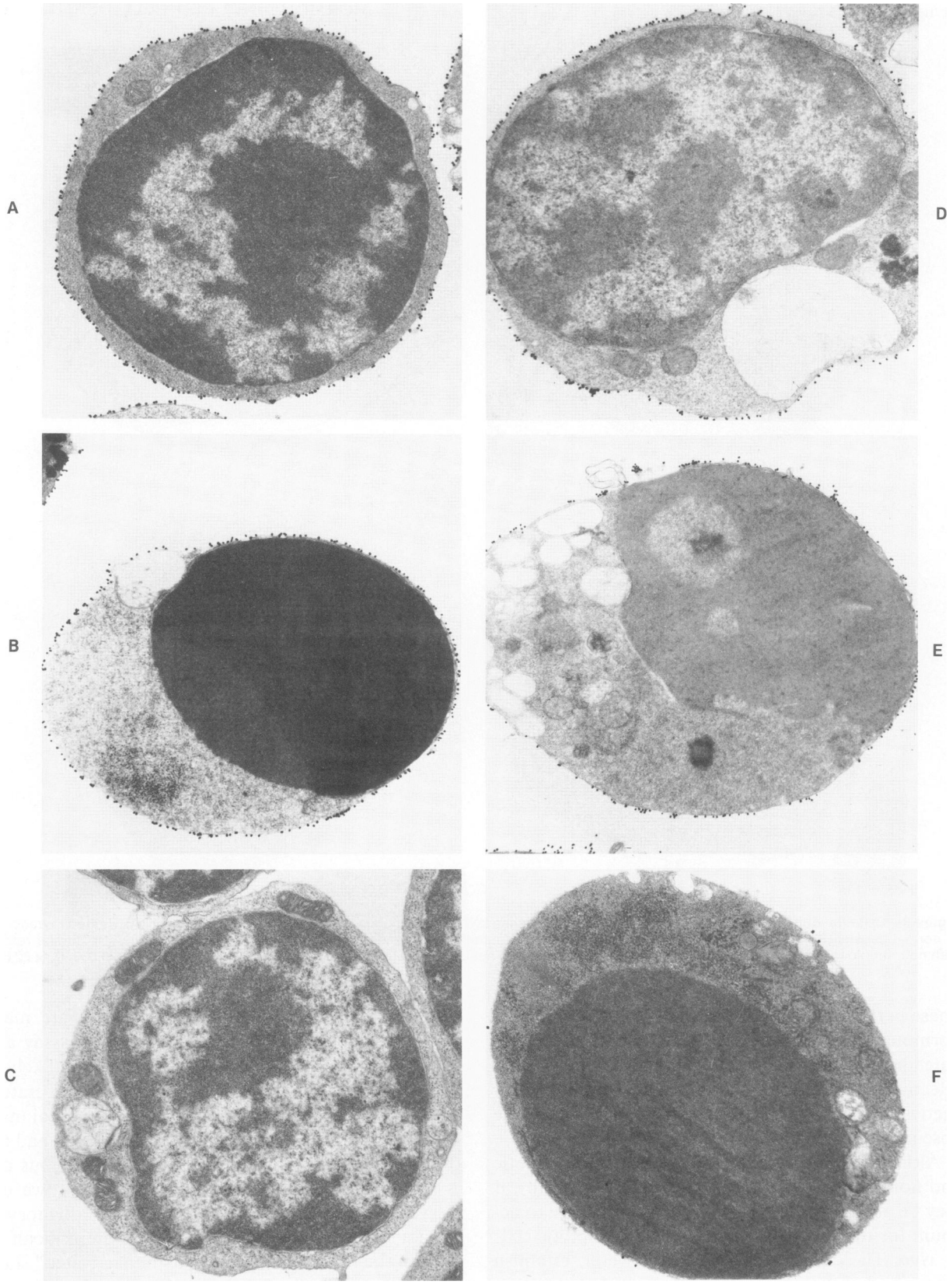


Figure 6—Gold-conjugated lectin binding by thymocytes. Cells of low density and normal morphology bind RCA I (**A**) and WGA (**D**) over most of their surface, although some "bare" patches are observed with WGA. (Occasional cells in the same preparations showed no WGA binding.) Apoptotic cells show indistinguishable binding patterns, both for RCA I (**B**) and WGA (**E**). Binding to RCA I is almost completely inhibited by coincubation with 200 mM galactose (**C**), and binding to WGA is similarly inhibited by N-acetyl-glucosamine (**F**). In this figure the morphologically normal cells were steroid-treated. Exactly analogous results were obtained with untreated cells. (Uranyl acetate and lead citrate, **A**, **D**, and **E**, ×18,000; **B** and **C**, ×14,000; **F**, ×26,000)

served to proceed to phagocytosis in the appropriate conditions (eg, incubation at 37 C), but the kinetics of phagocytosis will be the subject of a separate study.

The surface of apoptotic thymocytes lacks microvilli and is reduced in area, perhaps by the process of shedding multiple membrane-bounded subcellular fragments. It also becomes pitted, probably through fusion of cytoplasmic vesicles with the plasma membrane. Some of these features have also been noted in other cell types in which apoptosis is induced by glucocorticoids[24] and in thymocytes undergoing apoptosis following stimuli other than glucocorticoid treatment.[25] It is thus probable that they reflect basic mechanisms in the process of apoptosis.

Coincident with these gross morphologic changes, the surface charge density—revealed here indirectly by cell electrophoretic mobility[26]—was reduced in apoptotic cells. Partial desialylation may be responsible for this reduction, since the mobility of apoptotic and morphologically normal cells became identical following neuraminidase treatment. A deficiency in terminal NANA residues might be expected in the glycans of endoplasmic reticulum prematurely exposed on the cell surface by the blistering process we have observed in apoptosis, since terminal residues are inserted last in the process of membrane maturation.[27] Alternatively, desialylation of the membrane might be achieved by selective removal of terminal residues from otherwise normal sialylated glycan chains, or by removal of entire sialoglycoprotein molecules from the membrane, as appears to occur in aging erythrocytes.[28,29] Incomplete synthesis or terminal desialylation of glycan chains would alter the pattern of exposed sugar residues on the cell surface and, perhaps, afford a recognition signal to endogenous lectins on phagocytic cells. Even complete removal of surface glycoproteins might have the same effect, through "unmasking" glycoproteins previously deeper in the membrane. Endogenous lectins capable of recognizing desialylated glycoproteins both in solution[30] and on cell surfaces[31-33] have been identified in a number of cell types, including macrophages. That macrophage recognition of apoptotic cells is indeed mediated by this type of process is suggested by the inhibition of binding by N-acetyl glucosamine but not by D-mannose.

Using FITC-conjugated lectins with specificities both for sialic acid residues and sugars normally deep within the glycan chains, however, we have been unable to detect alterations in the exposed sugars on apoptotic cells. This was true even adjacent to sites where Golgi and endoplasmic reticulum membrane appeared to be fusing with the plasma membrane, although similar methods have proved capable of identifying focal alterations in membrane structures in other situations.[34] We were also unable to demonstrate selective quantitative changes in the W3/13 antigen, a heavily sialylated glycoprotein considered to be of major importance in the determination of cell surface charge density.[35] It appears that the majority of the molecules detected by our 8 probes are conserved in apoptosis. These negative results show that, despite the florid morphologic changes on the surface of the apoptotic cell, many major molecular determinants remain intact. The changes which permit recognition of apoptotic cells by macrophages appear to be selective and involve a minority of molecular species. Detailed description of these changes, in the isologous mouse system, is the subject of a further communication.

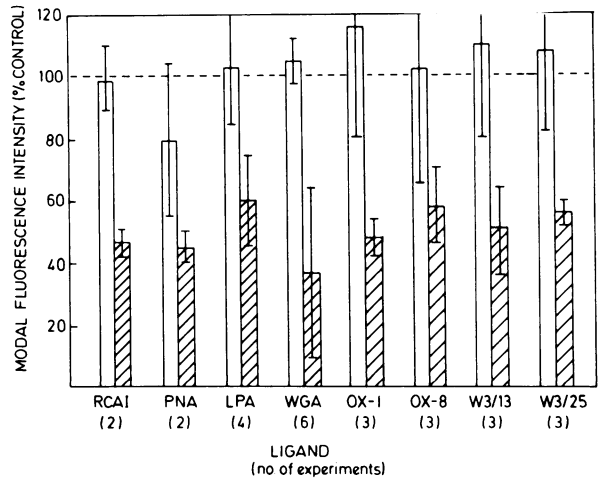


Figure 7—Fluorescence intensities of thymocytes after binding to lectins and monoclonal antibodies. The histogram shows modal fluorescence intensities (mean ± SE), for apoptotic cells (*hatched bars*) and low-density, steroid-treated cells (*open bars*), expressed as percentages of the modal intensity of the untreated cells in the same experiment. Fluorescence intensities of all cell types on binding to RCA I was reduced to <8% by coincubation with 200 mM galactose; similarly, PNA binding was reduced to <30% by N-acetyl galactosamine and to <4% by galactose; LPA binding was reduced to <6% by prior neuraminidase treatment; and WGA binding was reduced to approximately 30% by coincubation with N-acetyl glucosamine and to approximately 70% by neuraminidase.

References

1. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
2. Wyllie AH, Kerr JFR, Currie AR: Cell death: The significance of apoptosis. Int Rev Cytol 1980, 68:251–356
3. Sandow BA, West NB, Norman RL, Brenner RM: Hormone control of apoptosis in hamster uterine luminal epithelium. Am J Anat 1979, 156:15–36
4. Wyllie AH, Morris RG: Hormone-induced cell death: Purification and properties of thymocytes undergoing

apoptosis after glucocorticoid treatment. Am J Pathol 1982, 109:78–87
5. Searle J, Lawson TA, Abbott PJ, Harmon B, Kerr JFR: An electron-microscope study of the mode of cell death induced by cancer chemotherapeutic agents in populations of proliferating, normal and neoplastic cells. J Pathol 1975, 116:129–138
6. Kay MMB: Mechanism of removal of senescent cells by human macrophages in situ. Proc Natl Acad Sci USA 1975, 72:3521–3525
7. Czop J, Fearon DT, Austen KJ: Membrane sialic acid on target particles modulates their phagocytosis by a trypsin-sensitive mechanism on human monocytes. Proc Natl Acad Sci USA 1978, 75:3831–3835
8. Kay MMB: Isolation of the phagocytosis-inducing IgG-binding antigen on senescent somatic cells. Nature 1981, 289:491–494
9. Wyllie AH, Morris RG, Smith AL, Dunlop DJ: Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 1984, 142:67–77
10. Stuart AE, Habeshaw JA, Davidson AE: Phagocytosis in vitro, Handbook of Experimental Immunology. 3rd edition. Edited by DM Weir. Oxford, Blackwell, 1978, pp 31.1–31.30
11. Lachmann PJ, Hobart MJ: Complement technology,[10] pp 5A.1–5A.23
12. Bianco C, Griffin FM, Silverstein SC, Griffin JA: Studies of the macrophage complement receptor: Alteration of receptor function upon macrophage activation. J Exp Med 1975, 141:1278–1290
13. Van Oss C, Fike RM, Good RJ, Reinig JM: Cell microelectrophoresis simplified by the reduction and uniformization of the electro-osmotic backflow. Anal Biochem 1974, 60:242–251
14. Vladutiu GD, Fike RM, Amigone VT: Influence of sialic acid on cell surface properties in I-cell disease fibroblasts. In Vitro 1981, 17:588–592
15. Horisberger M, Rosset J: Colloidal gold, a useful marker for transmission and scanning electron microscopy. J Histochem Cytochem 1977, 25:295–305
16. Roth J, Binder M: Colloidal gold, ferritin and peroxidase as markers for electron microscopic double labelling lectin techniques. J Histochem Cytochem 1978, 26:163–169
17. Gasic G, Berwick L: Hale stain for sialic acid-containing mucins: Adaptation to electron microscopy. J Cell Biol 1963, 19:223–228
18. Pearse AGE: Histochemistry: Theoretical and Applied. 3rd edition. Vol 2. London, Churchill, 1972, p 1444
19. Burger MM, Goldberg AR: Identification of a tumor-specific determinant on neoplastic cell surfaces. Proc Natl Acad Sci USA 1967, 57:359–366
20. Liebovich SJ, Knyszynski A: In vitro recognition of "old" red blood cells by macrophages from syngeneic mice: Characteristics of the macrophage-red blood cell interaction. J Reticuloendothel Soc 1980, 27:411–419
21. Hurle JM, Lafarga M, Hinchliffe JR: The surface coat of the embryonic limb mesenchyme cells during morphogenetic cell death: An ultrastructural study of the chick interdigital necrotic zones (INZ) using ruthenium red and concanavalin A. Exp Cell Res 1981, 133:465–469
22. Newman SL, Henson JE, Henson PM: Phagocytosis of senescent neutrophils by human monocyte derived macrophages and rabbit inflammatory macrophages. J Exp Med 1982, 156:430–442
23. Duvall E, Wyllie AH, Morris RG: On the recognition by macrophages of cells undergoing programmed cell death (apoptosis). (Manuscript submitted)
24. Galili U, Leizerowitz R, Moreb J, Gamliel H, Gurfel D, Polliack A: Metabolic and ultrastructural aspects of the *in vitro* lysis of chronic lymphocytic leukaemia cells by glucocorticoids. Cancer Res 1982, 42:1433–1440
25. Yamada T, Ohyama H: Changes in surface morphology of rat thymocytes accompanying interphase death. J Radiat Res 1980, 21:190–196
26. Brinton CC, Lauffer MA: The electrophoresis of viruses, bacteria and cells and the microscope method of electrophoresis. Electrophoresis. Vol 1. Edited by M Bier. New York, Academic Press, 1959, pp 427–492
27. Montreuil J: Primary structure of glycoprotein glycans: Basis for the molecular biology of glycoproteins. Adv Carbohyd Chem Biochem 1980, 37:157–223
28. Balduini CL, Singaglia F, Ascari E, Balduini C: Ageing of rabbit red cells in vitro: Membrane modifications and their possible role in red cell survival in vivo. Acta Haematol 1981, 65:263–269
29. Lutz HU, Fehr J: Total sialic content of glycophorins during senescence of human red blood cells. J Biol Chem 1978, 254:11177–11180
30. Hudgin RL, Pricer WE, Ashwell G, Stockert RJ, Morell AG: The isolation and properties of a rat liver binding protein specific for asialoglycoprotein. J Biol Chem 1974, 249:5539–5543
31. Weir DM, Ogmundsdottir HM: Nonspecific recognition mechanisms by mononuclear phagocytes. Clin Exp Immunol 1977, 30:323–329
32. Kolb H, Kolb-Bachofen V, Schlepper-Schafer J: Cell contacts mediated by D-galactose specific lectins on liver cells. Biol Cell 1979, 36:301–308
33. Nagamura Y, Kolb H: Presence of a lectin-like receptor for D-galactose on rat peritoneal macrophages. FEBS Lett 1980, 115:59–62
34. Roth J, Brown D, Orci L: Regional distribution of N-acetyl-D-galactosamine residues in the glycocalyx of glomerular podocytes. J Cell Biol 1983, 96:1189–1196
35. Brown WRA, Barclay AN, Sunderland CA, Williams AF: Identification of a glycophorin-like molecule at the cell surface of rat thymocytes. Nature 1981, 289:456–460

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