ENZYME-INDUCED MODIFICATION OF THE SURFACE PROPERTIES OF LYMPHOID CELLS IN MALIGNANT DISEASE
I. EFFECT OF TRYPSIN ON ROSETTE FORMATION BY LYMPHOCYTES IN MYELOMATOSIS

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Summary.—The surface properties of blood lymphocytes from treated myeloma patients and healthy controls were studied in vitro. The patients were tested 6 weeks after the last treatment to allow time for cells to recover from possible drug toxicity.

Peripheral-blood lymphocytes were tested for rosette formation with unsensitized sheep erythrocytes (E rosettes) and with complement and antibody-coated erythrocytes (EAC rosettes). The tests were duplicated using lymphocytes pretreated with trypsin. As others have noted, myelomatosis is associated with increased blood levels of EAC-rosette-forming cells and a marked reduction in E-rosette-forming cells. E-rosette formation was significantly increased by pretreatment of myeloma lymphocytes with trypsin. By contrast, enzyme-treated cells showed no significant change in EAC-rosette formation.

These results suggest that the absolute number of circulating T cells is probably not reduced in myelomatosis, but that the surface of T cells is somehow modified so that a proportion of them lose the ability to form E rosettes.

Human T lymphocytes can be identified by their ability to form spontaneous rosettes with unsensitized sheep red cells (E rosettes) (Jondal et al., 1972; Minowada et al., 1972) and by lack of surface-membrane receptors for immunoglobulin or complement components (C3b or C3d). Human B lymphocytes can be identified by their ability to form rosettes with sensitized sheep erythrocytes (EAC rosettes) (Bianco et al., 1970; Jondal et al., 1972) and by the presence of receptors for aggregated immunoglobulin.

Previous reports, from this laboratory and from others, have shown that myeloma patients have low levels of E-rosette-forming lymphocytes, and a preponderance of EAC-rosette-forming lymphocytes (Mellstedt et al., 1973; Jones & McFarlane, 1975). However, it is not known whether the subnormal levels of E-rosette-forming cells were the consequence of a reduction in the absolute number of circulating T lymphocytes or of changes in the surface properties of this population of cells which prevent them from forming E rosettes in vitro.

It has recently been reported that pretreatment of normal peripheral blood lymphocytes (PBL) with neuraminidase significantly enhanced E- and EAC-rosette formation (Galili & Schlesinger, 1974; Han & Minowada, 1976). The number of EAC rosettes slightly increased when PBL from patients with chronic lymphocyte leukaemia or acute lymphoblastic leukaemia were pretreated with Vibrio cholera neuraminidase (Han & Minowada, 1976).

The present study was designed to in-

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vestigate the effect of trypsin treatment on E- and EAC-rosette formation by PBL from myeloma patients.

MATERIALS AND METHODS

Patients and controls.—The investigations were carried out on 30 patients for whom the diagnosis of myelomatosis had been well established. This was based on the following laboratory findings: (1) plasma-cell infiltration of marrow, as evidenced by the plasma cells constituting more than 15% of the total cell population of the aspirated marrow; (2) presence of a monoclonal protein in the serum and concentrated urine, as demonstrated by cellulose acetate electrophoresis, with a reduction in non-myeloma serum immunoglobulins; and (3) in most cases the presence of erosive changes in the skull, vertebral column and pelvic bones as shown on X-ray films. All the patients had received several courses of the intermittent combination chemotherapeutic regime previously described by Azam & Delamore (1974).

Healthy blood donors with normal lymphocyte counts (1000–4000/mm³) and receiving no drugs, were studied as controls.

Collection of blood specimens.—Venous blood specimens were collected in preservative-free heparin under sterile conditions. In the case of myeloma patients, the following protocol was adopted for blood collection. Each course of treatment lasted 14 days followed by a rest period of 6 weeks, thus allowing sufficient time for marrow recovery from the previous therapeutic course. Blood samples were collected from the patients before the next course of treatment.

Lymphocyte preparation.—Lymphocytes were separated from freshly collected venous blood, under sterile conditions, by density-gradient sedimentation in Ficoll–Triosol solution (Böyum, 1968). The cells were washed ×3 and the pellet suspended in Dulbecco’s phosphate-buffered saline (PBS). Aliquots from each lymphocyte suspension were smeared and stained with Leishman stain. The cell suspensions used in all experiments were free of red cells and composed of >95% small lymphocytes. The viability of lymphocytes as revealed by the Trypan-blue dye exclusion technique was >99% in all experiments.

Enzyme.—A stock concentrated solution of trypsin, 2·5% in calcium- and magnesium-free PBS, was purchased from Flow Laboratories U.K. Its activity was checked with N-benzoyl-L-arginine ethyl ester in phosphate buffer (pH 7·6) as substrate.

Trypsinization of lymphocytes.—Separated lymphocytes were resuspended in PBS at 10⁶ cells/ml and each suspension was divided into 2 or more equal fractions. After centrifugation the cell pellet was resuspended either in trypsin solution (10–500 µg/ml trypsin in PBS) or in PBS alone followed by incubation at 37°C for 30–120 min without agitation. The cell suspension was chilled on ice and centrifuged at 4°C (300 g, 10 min) to sediment the cells. The supernatants were removed, the cell pellets were washed twice in PBS at 4°C and finally resuspended in complete nutrient medium composed of RPMI 1640 medium supplemented with 10% foetal calf serum (heat-inactivated) plus antibiotics. The recovery of lymphocytes after trypsinization was determined by total cell counting, using improved Neubauer’s chambers, before and after enzyme treatment, expressed as a percentage.

In all experiments, the recovery was >97% and the viability of both trypsin-treated and untreated cells was usually >90%.

T-cell rosettes (E-binding lymphocytes).—0·25 ml of trypsinized and control lymphocyte suspensions containing ~10⁶ cells in nutrient culture medium (RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum plus antibiotics) were allowed to form spontaneous rosettes with 0·25 ml of a 1% suspension of fresh, well washed sheep erythrocytes (E) prepared in the same medium. The mixture, contained in a wide flat-bottomed 15 × 41 mm polyurethane tube, was centrifuged at 200 g × 2 min and incubated at 4°C for 16 h.

B-cell rosettes (EAC-binding lymphocytes).—5 ml of washed sheep erythrocytes were resuspended in nutrient medium to a concentration of 5% (v/v) and mixed with an equal volume of rabbit antibody to sheep erythrocytes prediluted 1:2000 in culture medium and incubated at 37°C for 30 min (Mellstedt & Holm, 1973). After the incubation the cells were washed ×3 and resuspended in 5 ml of culture medium. An equal volume of fresh human serum diluted 1:20 with supplemented RPMI 1640 medium, as source of complement, was added and further incubated at room temperature for 30 min. The anti-
body–complement-coated sheep erythrocytes (EAC) were finally washed ×3 and adjusted to 1% (v/v) cell suspension in nutrient culture medium. 0.25 ml of the EAC cells were mixed with 0.25 ml of lymphocyte suspension (trypsin-treated and untreated) in flat-bottomed polyurethane tubes. After gentle centrifugation (200 g, 5 min) and incubation at ambient room temperature for 4 h, the EAC rosettes were counted.

Before the rosettes were counted, most of the supernatant was discarded from each tube and the cells were gently tapped. Four hundred cells were counted under a sealed coverslip from each triplicate sample and lymphocytes binding more than 3 E or EAC erythrocytes were regarded as rosette-forming. The final result of each rosetting cell type was expressed as a percentage of absolute total cell population, modified from the method described by Dellom (1974).

RESULTS

Optimum trypsin concentration

The effect of exposing lymphocytes to varying concentrations of trypsin on the percentage of E-binding lymphocytes is shown in Fig. 1. The most striking finding was that the proportion of myeloma lymphocytes forming E rosettes increased with increasing trypsin concentration and reached the maximum level when the trypsin concentration was 300 μg/ml. Greater concentrations of trypsin (up to 500 μg/ml) reduced the mean percentage of E-rosette-forming cells. However, the mean percentage of trypsinized myeloma lymphocytes forming E rosettes remained significantly higher (P < 0.01) than the percentage for untrypsinized myeloma lymphocytes. By contrast, exposure of normal lymphocytes to trypsin caused a marked reduction in the mean percentage of E-rosette-forming cells. At 300 μg/ml trypsin, the percentage of E-rosette-forming cells was reduced to about 80% of that for untrypsinized normal lymphocytes. This was further reduced to 60% when the trypsin concentration was increased to 500 μg/ml. However, the mean percentage of trypsinized myeloma lymphocytes forming E rosettes was significantly lower than that of trypsinized normal lymphocytes (P < 0.02).

Optimum incubation time

The mean percentage of E-rosette-forming lymphocytes from myeloma patients and normal donor PBL before and after exposure to 300 μg/ml trypsin for varying incubation times at 37°C is presented in Fig. 2. The percentage of E-rosette-forming cells within the population of trypsinized myeloma lymphocytes increased steadily with time, reaching a peak at 90 min incubation. The mean percentage of untrypsinized myeloma cells forming E rosettes remained relatively con-
TABLE.—Statistical analysis: Effects of trypsinization on E and EAC rosettes formed by lymphocytes from myeloma patients and normal donors

| Subjects          | No. | E rosette       | EAC rosette       |
|-------------------|-----|-----------------|-------------------|
|                   |     | A               | B                 | A                | B                 |
| Controls          | 30  | 55·4±13·1a      | 34·0±12·4b        | 39·6±9·3c        | 36·1±8·0f         |
| Myeloma           | 30  | 17·1±11·8c      | 26·8±12·8d        | 56·0±12·9g       | 53·5±11·0h        |

Results are mean ± s.d. A = untreated and B = trypsin-treated peripheral-blood lymphocytes. Statistically analysed for significance by Student’s *t* test on paired samples and Wilcoxon matched signed rank test.

\( a \ vs \ b = P < 0·001; \ c \ vs \ d = P < 0·001; \ e \ vs \ f = P > 0·05; \ g \ vs \ h = P > 0·05; \ a \ vs \ c = P < 0·001; \ b \ vs \ d = P < 0·020; \ e \ vs \ g = P < 0·001; \ f \ vs \ h = P < 0·001. \)

Fig. 2.—Correlation between duration of trypsinization and the percentage of E rosettes formed. 300 μg trypsin/ml was used throughout. (△—△)=trypsin-treated myeloma lymphocytes, (●——●)=untreated myeloma lymphocytes, (□——□)=trypsin-treated normal donor lymphocytes, and (○——○)=untreated normal donor lymphocytes. Note that at 90 min there was maximum effect of trypsin on the myeloma PBL and that there was an inverse relationship between the trypsin-treated myeloma and trypsin-treated normal lymphocytes. After trypsinization of the myeloma cells, their ability to form E rosettes approached that of untrypsinized normal lymphocytes.

However, after 90 min incubation, the effect of trypsin on the myeloma PBL was maximal, and an inverse relationship existed between the trypsin-treated myeloma and trypsin-treated normal lymphocytes.

Exposure to trypsin: effect on E rosettes (T cells)

Having established optimal conditions of trypsin treatment for differentiating myeloma patients and blood donors in a limited number of cases, the effect of trypsinization on E rosettes of PBL from a larger group of myeloma patients and normal blood donors was studied (Fig. 3).
A slight reduction of EAC rosettes was seen when the PBL from myeloma patients or normal donors were pretreated with trypsin. The reduction was, however, not statistically significant. The EAC-rosette level of trypsinized and untrypsinized myeloma lymphocytes was significantly higher than that of trypsinized and untreated normal lymphocytes respectively ($P < 0.001$).

**DISCUSSION**

This investigation provides evidence that trypsinization of lymphocytes from treated myeloma patients increased the proportion able to form spontaneous rosettes with sheep erythrocytes. Under the same conditions trypsin depressed the numbers of lymphocytes from normal donors able to form E rosettes. An interesting aspect of the result is that the proportion of E-binding cells from the trypsin-treated myeloma lymphocyte population slightly but consistently exceeded that of normal lymphocytes pretreated with trypsin at concentrations above 200 $\mu$g/ml (Fig. 1).

The depressed E-rosette formation by trypsin-treated normal lymphocytes confirmed the suggestion that the sheep erythrocyte receptor sites on the lymphocyte surface membrane are sensitive to trypsin and may be protein in nature (Wybran et al., 1972). An alternative explanation is that the surface changes on normal lymphocyte membranes have been markedly altered by trypsin, so that the electrostatic attraction between the lymphocyte and sheep erythrocyte had been reduced.

Although the exact mechanism of trypsin-induced enhancement of myeloma lymphocyte capacity to form spontaneous rosettes with sheep erythrocytes is not clearly understood, it is most likely to be related to changes in the surface-membrane properties of the cells following trypsinization. Acute-phase proteins (e.g. C-reactive protein) which are considerably increased in malignancy, suppress several

**Exposure to trypsin: effect on EAC rosettes (B cells)**

The comparative effect of trypsinization on rosette formation with sensitized sheep erythrocyte by normal and malignant PBL is presented in Fig. 4 and the Table.
functions of T lymphocytes (Mortensen et al., 1975). It is conceivable that the receptor sites on the surface membrane of malignant lymphoid cells were masked by serum factors which may be elaborated by the cells or absorbed on to the cell surface, and that trypsinization relieves this inhibition. Identification of the surface-membrane trypsinates from myeloma lymphocytes by double immunodiffusion showed the presence of C-reactive protein, α1-antitrypsin and α2-macroglobulin (unpublished data).

Augmentation of E-rosette formation by lymphocytes from myeloma patients following trypsinization suggests that T lymphocytes were present, and that their responsiveness can be partially or completely restored. Furthermore, the E-rosette formation which has been widely used as a means of determining the total T-cell population in patients with various types of malignant diseases (Mellstedt et al., 1973; Nemoto et al., 1974; Jones & McFarlane, 1975) may in certain circumstances provide inaccurate estimates of the absolute number of circulating T lymphocytes. In these cases the technique should be extended by the use of enzymes.

The effect of acute-phase proteins on surface-membrane-associated functions and properties of normal lymphocytes is currently being investigated.

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