Peripheral blood lymphocytes from some of patients with mycosis fungoides disease showed decreased ability to form rosettes with sheep erythrocytes. This decreased percentage of E-rosette forming cells could be normalized when those cells were incubated in culture for 20 hr. Since these data led us to considering a possible inhibitory factor present in patients' sera, we tested their ability to inhibit E-rosetting by T lymphocytes from normal donors, and found that sera from mycosis fungoides patients with low levels of E-rosetting blood lymphocytes showed greater inhibitory effect on E-rosette formation by normal T cells when compared to those either from normal donors or from mycosis patients who had almost normal levels of E-rosetting blood lymphocyte number. The E-rosette inhibitory factor was sensitive to 2-mercaptoethanol treatment and was copurified with serum IgM by ammonium sulfate precipitation and by sequential gel filtrations, suggesting that it might be an anti-T lymphocyte antibody naturally occurring during the disease process.

It has been reported that mycosis fungoides (MF) is one of the T cell malignancies [1-3], which usually undergoes 3 different stages, i.e., eczematous, plaque, and tumor stages. However, D'Emblee type occurs at the tumor stage and has poorer prognosis [4].

Recently, Nordqvist and Kinney [5] has reported that the MF patients have decreased percentage of peripheral blood lymphocytes (PBL) rosetting with sheep red cells (E). In the present study, we have tested E-rosette formation of PBL from the MF patients and have found that a certain serum factor might be responsible for the lowered number of E-rosetting PBL in the MF patients. This factor might be a naturally occurring anti-T cell antibody because it was sensitive to 2-mercaptoethanol treatment and was copurified with serum IgM by using ammonium sulfate precipitation and sequential gel filtrations.

**MATERIALS AND METHODS**

**Patient Selection and Staging**

Studies were performed in 5 MF patients varying in stages (Table I), who were collected between 1978 and 1979. The diagnosis was made by clinical criteria as well as by histological examinations.

**Lymphocyte Separation**

Mononuclear cells were separated from peripheral blood by centrifugation on a Ficoll-Conray gradient (d = 1.080) at 250 g for 20 min. Cells at the interface were collected, washed 3 times with phosphate-buffered saline (PBS) and suspended in RPMI-1640 medium. More than 98% of cells were viable after washing, as determined by trypan blue dye exclusion test [6].

**E-rosette Formation**

The ability of PBL to form spontaneous rosettes with neuraminidase-treated sheep E was examined by the method of Bentwich et al [7]. Briefly, equal volumes (0.1 ml) of 0.5% E and PBL (5 x 10^6/ml) were admixed and incubated at 37°C for 5 min. The mixture was then spun at 200 g for 5 min and kept at 4°C for 1 hr. The cells were gently resuspended and the percentage of cells with more than 2 sheep E were counted.

**E-rosette Inhibition Test**

The ability of sera from MF patients to inhibit E-rosette formation of PBL from normal donors was studied by the method previously described [8]. Briefly, 0.1 ml of normal PBL (5 x 10^6/ml) mixture was admixed and incubated at 37°C for 30 min. The mixture was then washed 3 times with PBS, suspended in 0.1 ml of RPMI-1640 and mixed with 0.1 ml of 5% sheep E. The mixture was centrifuged and incubated at 4°C for 1 hr. The numbers of E-rosetting cells were counted, estimating the percentage of E-rosette inhibition (% EI) as:

\[
\text{ Untreated %RFC - Serum-treated %RFC } \times 100 (\%)
\]

**Ammonium Sulfate Precipitation**

Six volumes of human serum were admixed with 4 vol of saturated ammonium sulfate solution (PH 7.0). Precipitates which contained gammaglobulin were collected by centrifugation at 10,000 g for 15 min. The supernatant was collected and dialyzed against PBS. The pellet was dissolved in an appropriate amount of PBS and reprecipitated with 40% NH4SO4. The precipitates were dissolved in PBS and dialyzed against PBS for 24 hr in a cold room.

**Treatment of Human Gamma-globulin with 2-mercaptoethanol (2-ME)**

Crude gamma-globulin (20 mg/ml) prepared by ammonium sulfate precipitation of undiluted human serum was mixed with 2-ME to give a final concentration of 0.05 M, which was a nontoxic dose for human PBL. After incubation at 20°C for 1 hr, 2-ME-treated samples were utilized for E-rosette inhibition experiments.

**Gel Filtration**

A column (1.8 x 50 cm) was prepared with either Bio-Gel A1.5m or Bio-Gel A5m (Bio-Rad Laboratories, Richmond, Ca.) and equilibrated with a running buffer of PBS (PH 7.4). Descending chromatography was performed at 4°C with a flow rate of 2 ml/hr. The column was calibrated for molecular weight estimation with purified human IgG, bovine serum albumin (BSA) and ovalbumin (OVA).

**Cytotoxicity Test**

Complement-dependent cytotoxicity was determined by trypan blue dye exclusion test using normal rabbit serum as a source of complement [6]. Equal volumes of target T cell suspension (5 x 10^6/ml), normal or MF patients' serum and complement (C) were mixed and incubated at 37°C for 30 min. Lysed cells were judged by uptake of trypan blue,
Estimating the cytotoxic index (CI) as

\[
\text{CI} = \frac{\% \text{ alive in C control} - \% \text{ alive in experiment}}{\% \text{ alive in C control}} \times 100 \%
\]

**Protein-A Rosette Assay**

Protein-A (Pharmacia Fine Chemicals, Upplands, Sweden) was coupled to rabbit red cells with chronic chlorride according to Johnson's method [9]. T cells were purified from PBL of normal donors by E-rosette depletion technique [10]. One hundred µl of T cell suspension (5 x 10^6/ml) were mixed with equal volume of IgM rich fraction (20 mg/ml) separated from MF patients' sera by using ammonium sulfate precipitation and Bio-Gel A1.5m gel filtration and were incubated at 4°C for 1 hr. After washing 3 times with PBS, the cells were suspended in 0.1 ml of RPMI-1640 and mixed with rabbit anti-human IgM anti-serum. The cells were incubated at 4°C for 30 min, washed 3 times with PBS and suspended in 0.1 ml of RPMI-1640 medium. Equal volumes (0.1 ml) of the cell suspension and 1% indicator red cells coated with protein-A were then admixed and spun at 200 g for 5 min. After 30 min at 4°C, the cells were gently suspended and the percentage of IgM-binding cells which were rosetted with rabbit erythrocytes coated with protein-A was counted.

**RESULTS**

**E-rosette Formation of PBL from MF Patients**

PBL were collected from 5 MF patients and their ability to form E-rosettes was compared before and after culturing them for 20 hr in RPMI-1640 medium with 10% fetal calf serum (FCS). As can be seen in Table II, depressed E-rosetting ability of MF patients' PBL, especially those from case 1 to 3, had recovered after the cultivation, showing almost normal levels of %E-RFC.

**Effect of Sera from MF Patients on E-rosette Formation of Normal PBL**

Since the data described above led us to consider a possible serum factor that can suppress E-rosette formation of human PBL, we tested the ability of MF patients' sera to interfere with E-rosette formation of PBL from allogeneic normal donors. Group A sera collected from MF patients (case 1-3) with low levels of %E-RFC showed E-rosette inhibition, whereas Group B sera obtained from MF patients (case 4 and 5) with almost normal levels of %E-RFC, as well as those from normal donors, had little effect on E-rosetting of normal PBL (Fig 1).

E-rosetting ability of PBL and E-rosette inhibitory effect of MF sera were compared in a single patient (case 1) who was diagnosed as D'emblee type and had a fatal prognosis. Sera were collected sequentially from the patient and the %E-RFC of his PBL blood cells counted simultaneously. As Fig 2 indicates, there was an inverse correlation between %E-RFC of the patient's PBL and the ability of the patient's serum to inhibit E-rosette formation.

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**Table I. Patient selection and staging**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Stage</th>
<th>WBC number</th>
<th>Cytogram (%)</th>
<th>Skin test</th>
<th>%E-RFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>Male</td>
<td>Tumor</td>
<td>14,200</td>
<td>18</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>Female</td>
<td>Tumor</td>
<td>6,800</td>
<td>15</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>78</td>
<td>Male</td>
<td>Plaque</td>
<td>9,900</td>
<td>10</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>Female</td>
<td>Plaque</td>
<td>3,500</td>
<td>25</td>
<td>+</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>Male</td>
<td>Plaque</td>
<td>7,000</td>
<td>28</td>
<td>+</td>
<td>28</td>
</tr>
</tbody>
</table>

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**Table II. E-rosette formation of blood lymphocytes from MF patients before and after culturing those cells for 20 hr**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>% E-rosette after culturing</th>
<th>% E-rosette before culturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
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<td>80</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>60</td>
</tr>
</tbody>
</table>

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*Percentage of E-rosetting lymphocytes in MF patients' blood was counted before and after culturing those cells in the presence of fetal calf serum.

**Partial Purification of E-rosette Inhibitory Factor from MF Serum**

Crude gamma-globulin fraction was prepared by 40% ammonium sulfate precipitation from MF and control normal human sera and its inhibitory effect on E-rosette formation of normal PBL was tested by E-rosette inhibition assay. While crude gamma-globulin fraction from normal human serum showed little effect on E-rosetting, the same fraction obtained from MF serum showed E-rosette inhibitory activity. This activity was lost when the gammaglobulin fraction of MF serum was treated with 2-ME (Table III).

The crude gamma-globulin fraction prepared from MF serum by ammonium sulfate precipitation was then separated by Bio-Gel A1.5m column chromatography into 2 different fractions (Fig 3). As Table IV shows, E-rosette inhibitory activity was found in the first fraction (F-1) but not in the second fraction of MF serum samples, whereas those fractions of normal human serum had no inhibitory activities. When those 2 fractions of MF serum were incubated with rabbit anti-human IgM and IgG sera in double immunodiffusion plates, we found that the first fraction contained IgM but not IgG, whereas the second fraction had IgG but not IgM (data not shown).

In the next experiment, the first fraction of Bio-Gel A1.5m effluent was further chromatographed by using Bio-Gel A5m column. As can be seen in Fig 4, the column effluent was separated into 4 different fractions, which were tested for E-rosette inhibitory activity and IgM content. Among these fractions, relatively high E-rosette inhibition was observed with the second fraction (Table V), where IgM was also copurified (Fig 5).

**Binding of IgM in MF Serum to T Cell Surfaces**

If the E-rosette inhibitory factor present in MF serum is an IgM antibody to human T cells, then one can expect that it might have cytotoxic effect on T cells. However, when we tested anti-T cell cytotoxic activity of IgM rich fraction (F-1) separated...
FIG 1. E-rosette inhibition induced by sera from MF patients and normal donors. MF sera were divided into 2 groups according to %E-RFC of MF patients’ blood lymphocytes. Group A from case 1–3 comprises sera obtained from MF patients with low level of %E-RFC, and group B from case 4 and 5 who had almost normal level of %E-RFC.

from MF serum by using 40% NH₄SO₄ precipitation and Bio-Gel A1.5m gel filtration, its cytotoxic activity was low, showing less than 20% CI against human T cells (Table VI).

Because of the insensitivity of cytotoxic testing, we then employed more sensitive technique for detecting IgM binding to human T cell surfaces. For this purpose, we used indirect protein-A rosette assay and could find that more than 40% of human T cells could specifically adhere to IgM from the fraction (F-1) separated from MF serum by ammonium sulfate precipitation and by Bio-Gel A1.5m filtration (Table VII).

### DISCUSSION

The possibility that a certain serum factor might be responsible for impaired E-rosette formation of T cells observed in MF patients [5] was suggested by the following observations; (1) depressed %E-RFC of MF patients’ PBL could be normalized by culturing them for 20 hr; (2) sera collected from MF patients suppressed E-rosette formation of PBL from normal donors. Moreover, we have noticed in this preliminary experi-
ment that there exists an inverse correlation between %E-RFC of PBL from MF patients and the ability of their sera to inhibit E-rosette formation of normal T cells. Thus, the sera from patients with low levels of %E-RFC showed greater E-rosette inhibitory activity than those sera collected from MF patients with almost normal levels of %E-RFC. This is also the case in a single MF patient, whose sera were sequentially collected and E-rosette inhibitory activities were compared to %E-RFC of this patient’s PBL at each time when the serum samples were collected.

Defective E-rosette formation of T lymphocytes has been observed in association with a variety of diseases including cancer [11], autoimmune disease [12], viral infection [13], sarcoidosis [14] and Hodgkin's disease [15]. In some of these cases, certain serum factors responsible for impaired E-rosetting have been reported [13–15]. Thus, Chisari and Edgington [13] have reported that beta-lipoprotein of the low-density lipoproteins of sera from patients with hepatitis B has E-rosette inhibitory activity. Menzoian et al. [16], on the other hand, have shown that immunoregulatory alpha-globulin can inhibit E-rosetting by T cells. In contrast to these studies, it has also been reported that E-rosette inhibitory serum factors are present in the gamma-globulin fraction in cases of Hodgkin's disease [15] and sarcoidosis [14]. The latter observation seems to be similar to our data indicating that the E-rosette inhibitory factor found in MF patients' sera was 2-ME-sensitive and could be copurified with serum IgM by using ammonium sulfate precipitation and gel filtrations. This factor might be an IgM antibody to T cells occurring naturally during the disease process but it has low complement-dependent cytotoxicity unlike natural antithy- 
cytotoxic antibody in SLE patients [12] or anti-T cell antibody found in sarcoidosis patients [14]. It is likely that E-rosette inhibitory factors detected in different diseases are heterogeneous and participate in immune dysfunction observed in such diseases.

The fact that MF patients who had low %E-RFC and high E-rosette inhibitory serum activity had poor prognosis, whereas those patients with normal levels of %E-RFC and little E-rosette inhibitory serum activity had better clinical courses suggests that measuring the serum inhibitory factor in M patients may provide a valuable tool for the confirmation of disease activity and prognosis in those patients. To confirm this possibility, further clinical study with a large number of MF patients is clearly required and the study is now being undertaken.

REFERENCES