Enhanced Binding of Neuraminidase-treated Sheep Erythrocytes to Human T Lymphocytes

By Michael S. Weiner, Celso Bianco, and Victor Nussenzweig

Pretreatment of sheep erythrocytes with neuraminidase greatly enhances their specific binding and rosette formation with human T lymphocytes. Since the rosettes dissociate at 37°C, a method was developed to isolate human T lymphocytes with a high degree of purity and good recoveries. Trypsin treatment of E abolishes their binding to T lymphocytes. Trypsin-treated erythrocytes can be coated with antibody and complement components and used as a specific reagent to detect the C3 receptor on human B lymphocytes, avoiding the risk of an interaction between the erythrocytes and T lymphocytes.

A LARGE PROPORTION OF normal human lymphocytes bind and form rosettes with sheep red blood cells (E).[1-4] The possibility that these lymphocytes (E-binding lymphocytes or EL) are thymus-derived was first suggested by the findings that most human thymocytes displayed this property[1,2] and that cells which bind E appear in the fetal thymus at 14 wk before they can be detected in the spleen, blood, and liver.[5] Further evidence for the thymic origin of EL can be summarized as follows: (1) Membrane receptors for modified C3, characteristic of most B lymphocytes (CRL),[6,7] are not detected on EL.[8] (2) The distribution of EL in lymphoid organs and in the peripheral blood of normal individuals or of patients with certain leukemias is complementary to that of B lymphocytes.[8-11] (3) There is still some controversy about the presence of immunoglobulin determinants on the membrane of EL. Wybran et al.[5] found that a rabbit antiserum to μ chains inhibited rosette formation, but other investigators have not confirmed this observation.[3,8,10] In addition, direct techniques such as radioautography and immunofluorescence with antisera to human κ, μ, and λ chains do not show membrane-bound Ig on EL.[8,9,12,13] (4) Antilymphocytic serum markedly inhibits rosette formation.[4,10,14] (5) Patients with Nezelof syndrome or with Wiskott-Aldrich syndrome have low numbers of EL.[5] These observations, as well as the recent finding that in a patient with Sézary's syndrome the vast majority of lymphocytes were EL,[8] underscores the usefulness of this membrane characteristic for the determination of the origin of lymphocytes in disease. The rosettes formed between EL and sheep erythrocytes are fragile, and this is probably one reason for the conflicting reports about the proportion of EL in normal peripheral blood. In this paper we studied the nature of the interaction between E and EL and found that pretreatment of E with neuraminidase greatly enhances rosette formation, while

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pretreatment of E with trypsin abolishes it. These findings allowed for the development of a more reliable method of detection of E-EL rosettes and for specifically isolating human peripheral blood T lymphocytes with good yields and purity.

MATERIALS AND METHODS

Heparin was purchased from Sigma Chemical Co., St. Louis, Mo. and diluted in saline to 1000 U/ml. Hank's balanced salt solution (10 ×, without $NaHCO_3$) from Grand Island Biological Co., Grand Island, N.Y., was diluted with distilled water and the pH adjusted to 6.5 (HBSS). RPMI 1640 was bought from Grand Island Biological Co., and Hepes buffer (Sigma Chemical Co.) was added to a final concentration of 10 m*M* and the pH adjusted to 7.4. Neuraminidase type VI from *Clostridium perfringens* was bought from Sigma Chemical Co., St. Louis Mo. Neuraminidase from *Vibrio cholera* was bought from Calbiochem, La Jolla, Calif. Trypsin and soybean trypsin inhibitor were purchased from Calbiochem, La Jolla, Calif.

Peripheral Blood Lymphocytes

Ten-twenty milliliters of venous blood was collected in a plastic syringe containing 100 μ of heparin, immediately diluted with equal volume of medium RPMI 1640, and layered on top of a Ficoll-Hypaque solution[8,15] density 1.076 g/cu cm at 25° C. The tube was centrifuged at 400 *g* (measured at the interface) for 35 min at room temperature. The cells at the interface were harvested, diluted at least five times with medium RPMI 1640, and washed twice by centrifugation at 200 *g* for 10 min. The purified cell suspension contained more than 90% of mononuclear cells. Tonsil lymphocytes were obtained from surgical specimens. The tonsils were enlarged and showed signs of chronic inflammation. They were teased in medium RPMI 1640 and washed twice by centrifugation. Trypan blue exclusion tests always showed more than 95% viable cells.

Trypsin Treatment of E and Preparation of E_TAC

Equal volumes of a 2-mg/ml trypsin solution and 5% E in RPMI 1640 were mixed and incubated at 37° C for 1 hr. At the end of the incubation period, an equimolar concentration of soybean trypsin inhibitor was added, and the cells were washed three times. E_TAC was prepared as described in Ref. 16 with rabbit anti-Forssman antibodies and mouse complement. The standard assay for E_TAC was performed as described in Ref. 16.

Neuraminidase Treatment of E (E_N)

Neuraminidase was diluted to 1 U/ml in HBSS pH 6.5 and 0.2 ml added to 1 ml of 5% E in the same buffer. The mixture was incubated for 1 hr at 37° C, then washed three times with RPMI 1640. Slight hemolysis occurred during incubation.

Depletion of CRL

Depletion of CRL was obtained by differential flotation of rosettes and free erythrocytes in Hypaque-Ficoll as previously described.[8]

Purification of E_N Rosettes

Purification of E_N rosettes was made by 1 *g* sedimentation of the mixture of cells containing rosettes and following the exact procedure described in Ref. 17. After sedimentation, only fractions containing more than 90% rosettes were pooled.

Standard Assay for the Detection of E_N-Binding Lymphocytes (E_NL)

In a 10 × 75-mm glass tube, 0.5 ml of a suspension containing 2 × 10^6 lymphocytes/ml was mixed with 0.5 ml of 0.5% E_N in RPMI 1640. The mixture was incubated at 37° C for 15 min, centrifuged at 200 *g* for 10 min at room temperature, then placed in an ice bath for 15 min (unless otherwise stated). The cells were gently resuspended by inversion of the tube several times. Rosettes consisting of a minimum of four red cells bound to a lymphocyte were counted in a hemacytometer. White cells were counted separately after lysis of the red cells in 1% acetic acid.

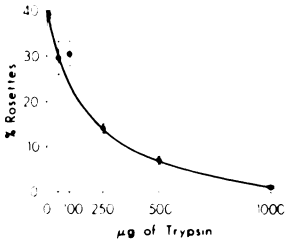

Fig. 1. Inhibition of rosette formation between E and human peripheral blood lymphocytes by pretreatment of E with trypsin. The red cells were incubated for 1 hr at 37° C with increasing concentrations of trypsin. At the end of the incubation period, equal weight of soybean trypsin inhibitor was added, and the cells washed by centrifugation. Each point represents the mean ± SE of three different experiments. In control tubes, soybean trypsin inhibitor was present since the beginning of the incubation of E with trypsin. These cells were found to bind to T lymphocytes as well as nontreated E.

The centrifugation at 200 *g* is critical since lower numbers of rosettes are found with lower centrifugal force.

RESULTS

Inhibition of Binding of E to Human T Lymphocytes by Pretreatment of the Erythrocytes With Trypsin (E_T)

Trypsin treatment diminished the ability of the erythrocytes to adhere to human lymphocytes, but relatively high concentrations of enzyme (1 mg/ml) were required for complete inhibition of binding (Fig. 1). Because the Forssman antigen is trypsin-resistant,[18] E_T could be subsequently treated with anti-Forssman antibody and complement components to prepare E_TAC for the detection of the complement (C3) receptor on the membrane of human B lymphocytes (CRL). The use of untreated E (to prepare EAC) as indicator cells may be hazardous in the assay of CRL if the test is performed at temperatures below 37° C,[2] because EAC may also interact with T lymphocytes by means of their receptors for E. Since E_TAC does not bind to T lymphocytes even if the cell remain in prolonged contact at 0° C, it is a better reagent.

Enhancement of Binding of E to Human T Lymphocytes by Pretreatment of the Erythrocytes with Neuraminidase (E_N)

Neuraminidase treatment of the red cells enhanced their ability to form clusters with human lymphocytes, and the degree of enhancement was dependent on the dose of enzyme (Fig. 2). Most rosettes were formed after 15 20 min of incubation at 0° C (Fig. 3), and their number sometimes decreased slightly if the incubation was prolonged. Neuraminidase from two different sources (*V. cholera* and *C. perfringens*) was equally effective. Heating neuraminidase from *V. cholera* for 10 min at 65° C[19] as well as incubating it with

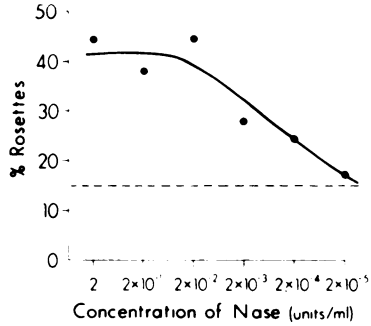

Fig. 2. Enhancement of rosette formation by treatment of E with Clostridium perifringens neuraminidase (E_N). Aliquots of E (10^9 cells) were preincubated for 1 hr at 37° C with increasing concentrations of enzyme and washed by centrifugation. Human mononuclear cells were incubated with the different E_N for 10 min at 37° C, centrifuged at 200 *g* for 10 min, and reincubated at 0° C for 15 min. After resuspension of the pellet, rosettes and leukocytes were counted separately. The dotted line represents the percentage of rosettes using untreated erythrocytes.

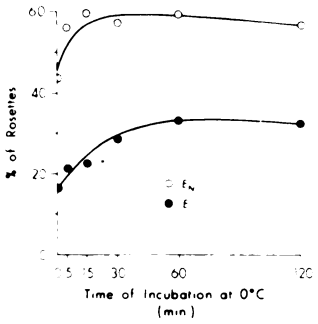

Fig. 3. Influence of time of incubation at 0° C on rosette formation with E_N which were prepared by incubation with 0.2 U/10^9E of neuraminidase from C. *perfringens* for 30 min and washed. The test was performed as explained in legend of Fig. 1. Pellets were resuspended after different times of incubation at 0° C, and rosettes counted.

red cells in the presence of chelating agents (Na_3HEDTA, 0.01 *M* final concentration)[20] abolished its activity.

The proportion of lymphocytes from normal human peripheral blood of ten donors which formed rosettes with E_N was 61.1% ± 8.3% (SD). Appropriate corrections for the contaminating granulocytes and monocytes were made. We had previously shown that only about 25% formed rosettes after 1 hr of incubation with E.[2] The donors in both studies were adults of both sexes, between 20 40 yr of age. In view of the greatly increased number of rosettes formed, the specificity of the interaction was carefully reevaluated, since it was possible that under the new conditions other leukocytes (including perhaps B lymphocytes) might bind E_N. The experiments below show that the features of the interaction between E or E_N and the leukocytes are very similar, and also that the clusters are formed mainly or exclusively around human T lymphocytes: (1) E_N or E bind to lymphocytes and not to monocytes or polymorphonuclear cells. The reaction is temperature dependent.[2] Thus, rosettes are not formed if cells are kept at 37° C or if the red cells and leukocytes are cooled at 0° C before they are mixed, and centrifuged and maintained at 0° C. In addition, rosettes formed with E_N or E dissociate at 37° C (2) Rosette formation with E_N or E[9,10] is not inhibited by the presence of antibodies to human lg (anti-κ, μ, and γ) or of 0.01 *M* Na_3HEDTA (pH 7.55) in the incubation medium. Jondal et al.[9] reported that E-EL interaction is inhibited by EDTA. Since rosette formation is very sensitive to pH changes, it is possible that their results were due to insufficient buffering capacity of the incubation medium and a drop in pH after the addition of the chelating agent. Recently, Ross et al. also reported that E-EL binding occurs in the presence of EDTA at pH 7.2.[13] (3) E or E_N do not bind to lymphocytes from certain cell lines (8866, Raji) which have been previously characterized as B by the presence of the C3 receptor or membrane-bound immunoglobulin (unpublished observation). (4) The EL population does not appear to contain lymphocytes which have membrane receptors for C3 (CRL), since the specific depletion of CRL was accompanied by a significant increase in the frequency of E_N rosettes among the remaining cells (Table 1). The values were quite close to those theoretically expected assuming that no cells which bind E_N were eliminated by this procedure. The method of depletion of CRL has been previously described[8] and is based on the separation of CRL-EAC rosettes from free leukocytes by differential flotation. In these experiments E_TAC instead of EAC were used in order to avoid the possibility that during

Table 1. Enrichment of E_N-Binding Lymphocytes in a Population of Human Tonsil Lymphocytes after Depletion of CRL*

Cells	Cell Recovery After Centrifugation (% of Loaded)	Distribution of Cells After Centrifugation (%)		Identification of Cells in Supernatant	
		Pellet	Supernatant	CRL (%)	E_NL (%)
Depleted	87.0	59.8	40.2	6.0	43.0
Nondepleted	86.6	19.8	77.2	54.8	22.6

*Tonsil lymphocytes were incubated with E_TAC, and most of the rosettes which were formed were pelleted by centrifugation on a Hypaque-Ficoll gradient. Controls (nondepleted) were similarly treated with E_T (instead of E_TAC).

incubation with the sensitized erythrocytes some T lymphocytes might interact with E. (e) Finally, lymphocytes which bind E_N (E_NL) do not contain, or contain very few, CRL. E_NL were specifically isolated by inducing cluster formation between E_N and lymphocytes from human tonsils or peripheral blood and separating the rosettes by sedimentation of the mixture at 1 *g* as described in Ref. 17. The yield was about 30% and purity above 90%. After purification, the red cells were eluted from the leukocytes by incubation at 37° C and frequent agitation in a Vortex mixer. At this stage, an excess of freshly prepared E_TAC was added to an aliquot of the cells, and the mixture of E_N, E_TAC, and leukocytes was rotated at 37° C for 30 min in order to detect the CRL among the isolated leukocytes. The assay for CRL was performed in the presence of the free E_N eluted from the rosettes because preliminary experiments has shown that they do not interfere with the binding of E_TAC to CRL. The percentage of E_N rosettes was determined according to the standard assay. Analysis of the results of Table 2 demonstrates that the population of lymphocytes which sedimented within the clusters contained very few CRL. The fraction of purified rosettes had 6.7% of contaminating free leukocytes. Since 55% of CRL were detected among the initial population of tonsil lymphocytes, 3.7% of CRL should be present among the leukocytes from the rosette-rich fraction (55% of 6.7%), and 5% were found.

In summary, the evidence above in conjunction with the previous findings presented in the introductory section strongly suggests that E_NL are part of the T-cell population.

Table 2. Isolation of E_N-Binding Lymphocytes (E_NL) by Sedimentation of Rosettes in the Earth's Gravitational Field (1 *g*)*

Source of Lymphocytes	Characterization of Cells Before Fractionation (%)		Nature of Cells in the Rosette-Rich Fraction (%)	
	CRL	E_NL†	CRL	E_NL
Peripheral blood	12.0	36.3	1.5	93.0
Tonsils	55.0	11.2	5.0	93.0

*Rosettes prepared with E_N were transferred to a sedimentation funnel, and the separation of clusters was performed as described in Ref. 17.

†Percentages not corrected for contaminating granulocytes and monocytes.

Table 3. Loss of Part of the CRL Population after Depletion of E_N-Binding Cells from Neuraminidase-treated Tonsil Lymphocytes*

Treatment of Tonsil Lymphocytes	Composition of Fractions Obtained After Rosette Formation With E_N and Centrifugation in Hypaque-Ficoll		
	Distribution of Recovered Cells (%)		CRL loaded × 100 CRL Recovered in Supernatant
	Pellet	Supernatant	
None	12.2	87.8	99.0
Neuraminidase	55.7	44.3	56.7

*Tubes containing 10^6 lymphocytes, 5 × 10^7 E and 0.2 U of neuraminidase/ml were incubated for 1 hr at 37°C, centrifuged at 200 *g* for 10 min at room temperature, reincubated at 0°C for 15 min for rosette formation. The resuspended cells were pooled and placed on top of a Hypaque-Ficoll solution and centrifuged. Supernatants contain free lymphocytes. Pellets contain rosettes and free erythrocytes. Controls were done in a similar way, but only the erythrocytes were pretreated with neuraminidase.

Treatment of Both Leukocytes and E With Neuraminidase

When both human leukocytes and sheep erythrocytes were treated with the enzyme before incubation for rosette formation, an additional increase in the number of clusters was obtained, and in some instances virtually all lymphocytes adhered to red cells. The results of several experiments (Table 3) demonstrate that after neuraminidase treatment both B and T lymphocytes bind E_N. Tonsil lymphocytes, treated or nontreated with neuraminidase, were incubated with E_T for rosette formation. More than 95% of the cells were viable by trypan-blue exclusion tests. After pelleting the rosettes by centrifugation on Hypaque-Ficoll, the proportion of CRL was determined among cells remaining in the supernatant. Ninety-nine per cent of CRL were recovered in tubes containing untreated lymphocytes, but only 56% were recovered from the supernatant of neuraminidase-treated cells. Thus, after removal of sialic acid, E_N also binds to the membrane of some neuraminidase-treated B lymphocytes.

DISCUSSION

The binding and rosette formation between E and human lymphocytes were greatly enhanced by pretreatment of the erythrocytes with neuraminidase. That the enzyme itself and not some contaminating material determined the enhancement in rosette formation was indicated by the observation that enzymes from two different sources (*V. cholera* and *C. perfringens*) were equally effective. Moreover, some inhibitors of neuraminidase from *V. cholera* abolished the effect. Maximum enhancement was observed when 10^9 red cells were incubated for 30 min with 0.2 U of neuraminidase, but an increase in rosette formation was noticeable when the red cells were treated with as little as 0.002 U of the enzyme. The mechanism through which neuraminidase treatment of E increases the formation of rosettes is not known. The simplest explanation is that, similar to the facilitation of agglutination of some red cells observed after neuraminidase treatment,[21] the removal of sialic acid and consequent net electrical charge alteration[22] facilitates the interaction between the T-cell receptor and the site on the E membrane.

In addition, these experiments show that the E site for T cell probably does not contain sialic acid, and therefore it is distinct from the heterophyle antibody

binding site of mononucleosis, which is destroyed by neuraminidase.[18] This is further suggested by the absence of inhibition of rosette formation by high concentrations of sialic acid (10 mg/ml) (unpublished observation). Although more and bigger clusters were formed when E_N instead of E were incubated with human leukocytes, no changes were observed in the known requirements for rosette formation such as, for example, the strict temperature dependency of the interaction. Moreover, complement-receptor lymphocytes (B cells) did not bind E_N as shown (a) by the experiments in which CRL were eliminated from a population of lymphocytes without altering the absolute number of cells which bound E_N, and (b) by specifically isolating lymphocytes which bind E_N and verifying that they do not contain CRL. Thus, it appears reasonable to conclude that the cells which bind E_N are the same as those which bind untreated E, that is, T lymphocytes.

Based on these findings a method was developed to isolate human T lymphocytes with a high degree of purity and good recoveries. It consists of purifying E_N rosettes by sedimentation in the earth's gravitational field, and subsequently separating the lymphocytes which are in the center of the clusters from the surrounding erythrocytes by reincubation of the rosettes at 37° C.

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