A NEW SURFACE MARKER ON T LYMPHOCYTES OF
HUMAN PERIPHERAL BLOOD*

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(Received for publication 5 July 1973)

Human peripheral blood lymphocytes are made up of two major populations,
characterized by functional differences and different cell surface markers. Lymphocytes
belonging to the bone marrow-derived population (B cells) have easily detectable im-
munoglobulin on their surface (1). In addition they have receptors for antigen/
antibody complexes or aggregated immunoglobulin (Fc receptors) (2) and receptors
for the complement component C3 (3). Human T cells may be detected by their
ability to form spontaneous rosettes with sheep erythrocytes (4). Plant lectins such
as concanavalin A (Con A) and phytohemagglutinin (PHA) bind to both B and T
cells but induce stimulation of DNA synthesis and blast transformation primarily in
the latter population (5). However, the latter property depends partly on the condi-
tions of stimulation, and B-cell stimulation by these agents has also been reported
(6). Mouse T cells are also characterized by their more pronounced anodic mobility
in the electric field, ascribable to their elevated concentration of surface-bound
N-acetyl neuraminic acid (7).

In contrast to Con A and PHA, the hemagglutinin from the snail Helix
pomatia (Helix pomatia A hemagglutinin) does not interact with untreated hu-
man blood lymphocytes as determined by binding experiments (8). In this study
we demonstrate (a) that human blood lymphocytes after treatment with neura-
mindase will bind Helix pomatia A hemagglutinin, (b) that the binding cells
most probably are T cells, and (c) that the binding properties of the cells can
be utilized for fractionation of lymphocyte subpopulations.

Materials and Methods

Human peripheral blood lymphocytes were obtained from the blood of healthy donors by
defibrination and gelatin sedimentation. After removal of phagocytic cells with colloidal iron
they were further purified by centrifugation through a layer of Ficoll-Isoaque (9). These pro-
cedures resulted in leukocyte preparations consisting to ≥ 98% of lymphocytes, containing
5-10% of contaminating erythrocytes. In this study, donors of blood group A were excluded
in order to avoid reactions between the hemagglutinin and contaminating A erythrocytes (8).

To create hemagglutinin receptors, purified lymphocytes were treated with neuraminidase
from Clostridium perfringens [type VI, 1-3 U/mg (NAN-lactose substrate), Sigma Chemical
Co., St. Louis, Mo.] at 37°C for 45 min. 25·10^6 lymphocytes in 1 ml of Tris-buffered Hanks’

* Supported by grant no. 2032-035 from the Swedish Natural Science Research Council.
We thank Dr. M. Jondal for lymphocyte fractionation on C3 columns and Mrs. E. S. Roberts-
son and Miss A. M. Duhrin for excellent technical assistance.
solution (TH) were mixed with 1 ml of TH containing 10 μg of neuraminidase. The cells were then spun down and washed once in TH (cell recovery 80-90%). Binding experiments were performed with cells in TH containing 0.1% human serum albumin (HSA). In the fractionation experiments the cells were suspended in N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES)-buffered medium RPMI 1640 (Bio Cult Laboratories, Paisley, Scotland) containing 0.1% HSA and cells were also stored in this medium at 4°C. Neuraminidase-treated cells were > 95% viable as assessed by vital staining with trypan blue. When incubated with PHA, stimulation of DNA synthesis was more pronounced than what was seen in the untreated controls (unpublished data).

Highly purified *Helix pomatia* A hemagglutinin was prepared by immunospecific purification on insolubilized hog A + H blood group substance (10). The material was homogenous by several physicochemical criteria and was completely precipitated by blood group A substance. The hemagglutinin has a mol wt of 79,000 ± 4,000 (11). It is made up of six identical or closely similar subunits, each containing one carbohydrate binding site. The binding site interacts most strongly with methyl-α-N-acetyl-d-galactosamine (Me-α-D-GalNAc), followed by methyl-α-N-acetyl-d-glucosamine (Me-α-D-GNAc) ≈ Me-β-D-GalNAc ≫ Me-β-D-GNAc > Me-α-D-galactose (10, 12-14).

For binding studies, the hemagglutinin was trace-labeled with carrier-free [125I]Na (Radiochemical Center, Amersham, England), using the chloramine-T procedure (8,15). The cell-binding experiments were performed with a constant number of cells and increasing amounts of labeled hemagglutinin. The binding data were treated according to Scatchard's derivation of the law of mass action. The calculated $K$ value is an apparent association constant reflecting the effect of multivalent interaction between hemagglutinin and cell. Earlier studies suggested that intact hemagglutinin is bound at least trivalently to human A erythrocytes (8).

In immunofluorescence experiments, the hemagglutinin was labeled with fluorescein isothiocyanate (FITC) on Celite (16). The fluorescein to protein molar ratio was 6.9. The experiments were carried out at room temperature with living cells in TH containing 0.02% NaN₃.

**RESULTS AND DISCUSSION**

We first studied the effect of neuraminidase treatment on the appearance of receptors for *Helix pomatia* A hemagglutinin. 25·10⁶ cells, incubated for 45 min with 10 and 100 μg of neuraminidase, respectively, bound approximately the same number of hemagglutinin molecules per cell at saturation (7·10⁶ and 11·10⁶, respectively) as determined by means of [125I]-labeled hemagglutinin. 1 μg of neuraminidase/25·10⁶ cells was not sufficient to create detectable numbers of hemagglutinin receptors during this incubation period. 10 μg/25·10⁶ cells was chosen for all subsequent experiments. Fig. 1 shows the binding curves for lymphocytes from two different donors and for neuraminidase-treated O erythrocytes from one of these. Assuming uniform binding to all cells, (see, however, below), each lymphocyte bound approximately 1·10⁶ hemagglutinin molecules and each erythrocyte approximately 0.3·10⁶ molecules. The binding curves are drawn as straight lines, calculated by the method of least squares. The apparent $K$ values were 5–7·10⁶ liter/mol for lymphocytes and 0.5·10⁶ liter/mol for erythrocytes, respectively. However, it should be noted that the binding curves for lymphocytes may not be linear. It would appear that the number of hemagglutinin receptors of neuraminidase-treated lymphocytes is similar to that of human A₁ erythrocytes (8). The apparent association constant is, however, two orders of magnitude lower. Assuming trivalent binding also to lymphocytes (8), these results suggest that the lymphocyte
structure responsible for binding of the hemagglutinin is not α-linked D-GalNAc as in the A determinant of the erythrocytes. The chemical nature of the lymphocyte receptor is not known. Possible candidates are nonreducing α-linked D-GalNAc or β-linked D-GalNAc (8, 10, 12–14). However, differences in spacing of the receptors or different influences of secondary binding forces may be responsible for the differences in avidity of binding.

Immunofluorescence studies of living, neuraminidase-treated lymphocytes from healthy donors, using FITC-conjugated hemagglutinin, demonstrated that only approximately 60% of the lymphocytes showed membrane fluorescence (Fig. 2 a). The percentage positively staining cells in lymphocyte preparations from five different blood donors (five experiments) varied from 58 to 73%. As seen from Fig. 2 a, increasing the conjugate concentration above a certain level did not increase the percentage of positive cells. The figure also shows that cells not treated with neuraminidase are negative and that prolonged neuraminidase treatment did not increase the percentage of positive cells. Staining is specific as demonstrated by inhibition with D-GalNAc and by the absence of significant inhibition with comparable concentrations of L-fucose (8).

Fig. 2 b shows the percentage of cells with positive membrane immunofluorescence after staining with FITC hemagglutinin of two lymphocyte preparations (two donors), enriched in T lymphocytes. Enrichment was accomplished by passage through columns filled either with glass beads or with Degalan beads (polymethylmeta acrylate, type W-26; Degussa Wolfgang AG, Hanau am Main, W. Germany) and charged with human immunoglobulin G and rabbit antihuman immunoglobulin (17) or with activated C3, respectively. Immunofluorescent staining for surface-bound immunoglobulin demonstrated that the passed preparations were deprived of B lymphocytes. Their content of lymphocytes with complement receptors was significantly reduced as indicated by their

1 Perlmann, P., H. Perlmann, and H. J. Müller-Eberhard. 1973. Manuscript in preparation.
strongly diminished capacity to form rosettes with complement-coated sheep erythrocytes (EAC) (3). Furthermore, the antibody-induced cytotoxicity of the passed cells against 51Cr-labeled chicken erythrocytes (Crbc) was very strongly reduced, thus indicating that the passed cell preparations were significantly depleted of cells with Fc receptors (9). As seen from Fig. 2 b about 90% of the passed cells were stained with FITC Helix hemagglutinin. In contrast, a sample of purified lymphocytes from a patient with multiple myeloma, containing a high percentage of B cells (56% as determined by rosette formation with EAC, 50% by immunofluorescent staining with anti-Ig), gave approximately 40% cells stained with FITC hemagglutinin. The results indicate that the lymphocytes that bind the hemagglutinin after treatment with neuraminidase belong to the thymus-derived variety.

In further studies we attempted to use the hemagglutinin to physically separate reactive and nonreactive lymphocytes. Degalan beads were coated with hog blood group A + H substance, washed, and packed in a column (0.5 × 14 cm) charged with different amounts of unlabeled hemagglutinin (supplemented with some 125I-labeled material) in TH. After extensive washing with TH, followed by RPMI 1640 HEPES (containing 0.1% HSA), lymphocyte suspensions were passed through the columns. These were immediately washed with medium, followed by elution with medium containing 0.1% D-GalNAc. All operations were performed with ice-chilled solutions. The passed and eluted cells were spun down and counted. In order to remove adsorbed hemagglutinin, they were subsequently washed with medium containing D-GalNAc. Radioactivity determinations demonstrated that less than 0.5 ng of hemagglutinin was left on any of the cell pellets after the D-GalNAc washing procedure. The
results of one experiment in which the column was charged with $16 \cdot 10^6$ lymphocytes are shown in Table I. As may be seen, the percentage of cells giving positive staining with FITC hemagglutinin was significantly lower in the passed fraction than in the fraction eluted with d-GalNAc, or in the cells passed through the control column, charged only with blood group substance. In other experiments in which the cells were tested for their capacity to form EAC rosettes, the percentage of complement receptor lymphocytes (CRL) in the passed cells was 40–50% as compared with 15–20% in the control. In contrast the d-GalNAc-eluted fraction was more or less completely depleted of CRL. As seen from Table I, the best separation was achieved with an intermediate concentration of hemagglutinin on the column. In this case the total cell recovery was approximately 50%. Incubation of the column with d-GalNAc for 5 min before elution increased the yield of FITC hemagglutinin-positive cells eluted from the column. We conclude that these procedures permit a separation of thymus-derived from thymus-independent lymphocytes in human peripheral blood. Further work is needed to increase the degree of separation.

**SUMMARY**

Neuraminidase treatment of human peripheral blood lymphocytes uncovers cell surface receptors that bind purified A hemagglutinin from the snail *Helix pomatia*. No hemagglutinin was bound to untreated lymphocytes. Binding studies with $^{125}$I-labeled hemagglutinin suggested that the number of receptors on neuraminidase-treated lymphocytes was approximately $1 \cdot 10^6$/cell. The apparent association constant for hemagglutinin binding to lymphocytes, as calculated from Scatchard's plots, was $5-7 \cdot 10^8$ liters/mol.

Immunofluorescent staining with FITC-conjugated hemagglutinin gave positive reactions with approximately 60% of the lymphocytes from normal donors. Positive staining was inversely related to the number of lymphocytes with Fc or complement receptors or with surface immunoglobulin, thus suggesting that

| Table I |
|----------------------------------|
| Fractionation of Neuraminidase-Treated Human Lymphocytes on Degalan Bead Columns Charged with Hog Blood Group A + H Substance and Helix pomatia A Hemagglutinin* |
| Beads charged with | Cells recovered | Cells staining with FITC hemagglutinin |
|-------------------|----------------|-------------------------------------|
| | Passed | d-GalNAc eluate | Passed | d-GalNAc eluate | |
| Hog A + H substance + 416 µg hemagglutinin | 1 | 23 | n.d. | 58 |
| " " + " " + 66 µg " " | 6 | 43 | 13 | 61 |
| " " + " " + 20 µg " " | 27 | 55 | 46 | 73 |
| Hog A + H substance | 90 | 0 | 73 | - |

* Packed Degalan beads were incubated with an equal volume of hog blood group A + H substance (0.5 mg/ml) for 18 h in the cold, washed extensively with TH, and packed in columns. The columns were charged with the indicated amounts of hemagglutinin and washed extensively before application of $16 \times 10^6$ lymphocytes. For other experimental details see text.

‡ n.d. = not done.
the lymphocytes with receptors for *Helix pomatia* A hemagglutinin are T cells. Cell fractionation on columns charged with hemagglutinin indicate that these receptors may be used for separating subpopulations of human peripheral lymphocytes.

REFERENCES

1. Wilson, J. D., and G. J. V. Nossal. 1971. Identification of human T and B lymphocytes in normal peripheral blood and in chronic lymphocytic leukaemia. *Lancet* 2:788.
2. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* 136:191.
3. Nussenzweig, V., C. Bianco, P. Dukor, and A. Eden. 1971. Receptors for C3 on B lymphocytes. Possible role in the immune response. *Prog. Immunol.* 1:73.
4. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. *J. Exp. Med.* 136:207.
5. Janossy, G., and M. F. Greaves. 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytohemagglutinins. *Clin. Exp. Immunol.* 9:483.
6. Phillips, B., and I. M. Reitt. 1973. Evidence for transformation of human B lymphocytes by PHA. *Nat. New Biol.* 241:254.
7. Nordling, S., L. C. Andersson, and P. Häyry. 1972. Thymus-dependent and thymus independent lymphocyte separation. Relation to exposed sialic acid on cell surface. *Science (Wash. D.C.)* 178:1001.
8. Hammarström, S. 1973. Binding of *Helix pomatia* A hemagglutinin to human erythrocytes and other cells. Influence of multivalent interaction on affinity. *Scand. J. Immunol.* 9:125.
9. Perlmann, P., H. Perlmann, and H. Wigzell. 1972. Lymphocyte mediated cytotoxicity *in vitro*. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* 13:91.
10. Hammarström, S., and E. A. Kabat. 1969. Purification and characterization of blood group A reactive hemagglutinin from the snail *Helix pomatia* and a study of its combining site. *Biochemistry* 8:2696.
11. Hammarström, S., A. Westtö, and I. Björk. 1972. Subunit structure of *Helix pomatia* A hemagglutinin. *Scand. J. Immunol.* 1:295.
12. Hammarström, S., and E. A. Kabat. 1971. Studies on specificity and binding properties of the blood group A reactive hemagglutinin from *Helix pomatia*. *Biochemistry* 10:1684.
13. Hammarström, S., A. A. Lindberg, and E. S. Robertsson. 1972. Precipitation of lipopolysaccharides from rough mutants of *Salmonella typhimurium* by an A hemagglutinin from *Helix pomatia*. *Eur. J. Biochem.* 25:274.
14. Hammarström, S. 1974. Structure, specificity, binding properties and some biological activities of a blood group A reactive hemagglutinin from the snail *Helix pomatia*. *Proc. N.Y. Acad. Sci.* In press.
15. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.)* 194:495.
16. Rinderknecht, H. 1962. Ultra rapid fluorescent labelling of proteins. *Nature (Lond.)* 193:167.
17. Wigzell, H., K. G. Sundqvist, and T. O. Yoshida. 1972. Separation of cells according to surface antigens by the use of antibody coated columns. *Scand. J. Immunol.* 1:75.