Surface Alterations in Calf Lymphocytes Oxidized by Sodium Periodate*

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In order to investigate alterations in surface structure in transformed lymphocytes, calf submandibular lymph node cell suspensions were oxidized with NaIO₄. Oxidized lymphocytes were morphologically transformed and had higher rates of DNA synthesis by 2 days after treatment. These results were prevented by reduction of the cell suspension with NaBH₄, or by neuraminidase treatment of cells prior to oxidation.

The amount of ¹²⁵I-labeled Agaricus bisporus lectin bound to cells immediately after oxidation and the affinity constant for binding were increased over 2-fold, while cells immediately following oxidation and reduction showed decreased receptors with still higher affinity for the lectin compared to untreated cells. The amount of Phaseolus vulgaris lectin bound to oxidized cells was also increased, but affinity was unchanged. Immediately following oxidation and reduction, these receptor sites were unchanged in number and affinity from untreated cells. In contrast, the number and affinity of receptors for concanavalin A were not changed immediately after oxidation or oxidation and reduction.

In order to define the extent of compositional changes in surface glycoprotein receptors, plasma membranes were isolated from frozen calf submandibular lymph nodes. Compared to untreated plasma membranes, oxidized membranes had similar contents of galactose, mannose, N-acetylgalactosamine, N-acetylgalactosamine, fucose, and amino acids. Sialic acid content of oxidized membranes was reduced when measured by thiobarbituric acid assay. Sialic acids of untreated plasma membranes co-chromatographed with N-glycolylneuraminic acid and N-acetylneuraminic acid, while those of oxidized membranes co-chromatographed with N-glycolylneuraminic acid and 5-acetamido-3,5-dideoxy-L-arabino-7-aldehyde-2-heptulosonic acid.

Therefore, specific surface conformational changes in certain classes of membrane glycoproteins are associated with mild Malapradian oxidation of membrane sialic acids. These temporally precede NaIO₄-induced transformation of calf lymphocytes. This is consistent with an hypothesis of membrane-mediated stimulation of lymphocyte transformation.

Initiation of cellular proliferation has been studied in lymphocytes transformed by a variety of stimuli, such as soluble antigens, mitogenic plant lectins, or histoincompatible lymphocytes. Recently, it has been shown that oxidation either by NaIO₄ (1, 2) or by neuraminidase plus galactose oxidase (3) can also increase DNA synthesis in cultures of mouse, rat, or human lymphocytes. Evidence suggests that transformation of mouse lymphocytes by NaIO₄ is specific for thymus-dependent lymphocytes (4). It has been proposed that both methods of oxidation (3, 5) result in transformation of the oxidized lymphocyte by formation of aldehydes from sialic acid or galactose, and that such aldehydes cross-link with other surface structures (perhaps free amino groups) to form a surface "lattice."

Lymphocytes that have been transformed by NaIO₄ provide an excellent system for investigating alterations in surface structure, since the cells have not been coated by foreign proteins such as lectins or antigens. Therefore, this investigation examined changes in surface structure of calf submandibular lymph node lymphocytes resulting from periodate oxidation, or oxidation plus NaBH₄ reduction.

EXPERIMENTAL PROCEDURE

Materials—Concanavalin A was purchased from Miles-Yeda (Kankakee, Ill.) and was extensively dialyzed against 0.15 M NaCl prior to

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use. The erythroagglutinating lectin of Phaseolus vulgaris (E.PHA) was purified from Bacto-phytohemagglutinin-P (Difco, Detroit, Mich.) by the method of Weber et al. (6) to apparent homogeneity as described by polyacrylamide gel electrophoresis. Agaricus bisporus, commercial mushrooms (Moonlight Mushrooms, Butler County Farm, West Winfield, Pa.) were purchased in a local supermarket. One of the lectins (mushroom PHA-B) was purified from these mushrooms by the method of Freest and Konold (7). Tritiated thymidine and NaBH₄, were obtained from New England Nuclear (Boston, Mass.) and NaBH₄ was purchased from Amersham/Seal (Arlington Heights, Ill.). Fetal calf serum and Medium 199 were obtained from Grand Island Biological Co. (Grand Island, New York). N-Acetylneuraminic acid and N-glycolyneuraminic acid were purchased as standards for assay and chromatography from Sigma (St. Louis, Mo.). Vicia fischeri neureaminidase was purchased from Calbiochem (La Jolla, Calif.).

Lymphocyte Suspensions—Fresh calf submandibular nodes were donated by Star Meat Packing Co. (St. Louis, Mo.). The lymph nodes were dissected free of connective tissue, washed in sterile 0.15 M NaCl, and then minced in a small volume of 0.15 M NaCl. After large tissue clumps had settled, lymphocytes in the supernatant fluid were centrifuged and then resuspended in 0.15 M NaCl. Binding studies were performed immediately after resuspension in 0.15 M NaCl.

Periodate oxidation was performed by incubating lymphocytes at a concentration of 20 × 10⁶ cells/ml in 0.4 M NaO₂/0.005 M sodium phosphate, pH 7.4/0.15 M NaCl for 15 min at 25°C in a lighted laboratory. This reaction was terminated by centrifugation and resuspension in Medium 199, and then in 0.15 M NaCl. Binding studies were performed immediately after resuspension in 0.15 M NaCl.

Periodate-oxidized lymphocytes were reduced in sodium borohydride by incubation at a concentration of 20 × 10⁶ cells/ml in freshly prepared 1 mm NaBH₄/0.005 M sodium phosphate, pH 7.4/0.15 M NaCl for 15 min at 25°C. The reaction was terminated by centrifugation and resuspension twice in Medium 199, and then in 0.15 M NaCl. Binding studies were performed immediately after resuspension in 0.15 M NaCl.

RESULTS

Characterization of Cell Lymphocyte System—Cell suspensions were made from fresh calf submandibular lymph nodes. Untreated cell suspensions contained 87% small, 9.5% medium, and 2% large lymphocytes, no lymphoblasts, and 1.5%
monocytes. After oxidation in NaIO₄, the cell suspension consisted of 62% small, 13% medium, and 25% large lymphocytes, no lymphoblasts, and 3% monocytes. The cells were 99% viable both before and after oxidation as determined by trypan blue exclusion, and 88% viable after NaBH₄ reduction. After 2 days in short term culture, lymphoblasts accounted for 2.1% ± 0.6% (1 S.E.) of cells in the untreated cell suspension, 21.3% ± 1.8% of cells in the oxidized cell suspension, and 4.8% ± 1.0% of cells in the oxidized and reduced cell suspension.

DNA synthesis was measured by tritiated thymidine incorporation into cultures of calf lymphocytes (Table II). Addition to the cultures of an optimal amount of Phaseolus vulgaris E-PHA, 15 μg/ml, or NaIO₄ oxidation of the cells resulted in an increased rate of DNA synthesis which was maximal at 48 hours. When NaIO₄, treatment was followed by NaBH₄ reduction, the rate of DNA synthesis was equal to that in untreated cells. NaBH₄ treatment alone resulted in the same rate of DNA synthesis as in untreated cells.

Lectin Binding to Lymphocytes— Untreated, oxidized, and reduced calf lymphocyte suspensions immediately after preparation were tested for their ability to bind 125I-mushroom PHA-B. One experiment is illustrated (Fig. 1). Oxidized cells bound more radioactive lectin than untreated cells. Oxidation plus reduction did not increase the lectin binding to cells. Although increased affinity of the lectin for the cells was more evident in the replicate experiments, apparent affinity of the lectin for the cells was slightly increased after oxidation, and further increased after oxidation plus reduction in the experiment illustrated (Fig. 1). Scatchard analyses of each of the three curves in this and other experiments with mushroom PHA-B indicated only one class of receptor (data not shown). This is also apparent in the double reciprocal plot (Fig. 1, right).

Results of five such binding studies are summarized in Table III. Oxidized cells had 2.6-fold more available receptor sites for mushroom PHA-B than untreated cells (p < 0.05). Lymphocytes which had been oxidized and reduced bound one-third less lectin than untreated cells (0.05 < p < 0.1). Affinity of mushroom PHA-B for receptor sites was enhanced 2.4-fold by oxidation of the cells (p < 0.05) and 5.6-fold by oxidation plus reduction (p < 0.05) compared to untreated cells.

A similar binding study using P. vulgaris E, 125I-PHA also suggested increased lectin binding to oxidized cells (Fig. 2).

### Table I

Characterization of lymphocyte subcellular fractions

| Fraction       | Protein | Sialic acid | DNA | 5'-Nucleotidase | Sucinic dehydrogenase | β-N-Acetylglucosaminidase |
|----------------|---------|-------------|-----|----------------|-----------------------|--------------------------|
|                | mg      | μmol        | nmol/mg | mg % | mg/μg | u mg | u mg | u mg | u mg |
| Crude Homogenate | 7000 | 100 | 53.5 | 100 | 8 | 1130 | 100 | 0.16 | 4500 | 100 | 0.64 | 1380 | 100 | 0.19 | 4630 | 100 | 0.67 |

* Specific activity in nmol/mg of protein.

### Table II

DNA synthesis in calf lymphocytes

| Treatment | Stimulation index | cpm/Culture |
|-----------|-------------------|-------------|
| None      | 1.0               | 116-261     |
| NaIO₄     | 19.4 ± 4.7        | 1639-2222   |
| NaIO₄ + NaBH₄ | 1.6 ± 0.5       | 144-1230    |
| P. vulgaris E-PHA | 10.6 ± 2.7 | 1636-1752  |

The binding of lectin to cells that had been oxidized and reduced was similar to that with untreated cells. The slight difference in association constant suggested in Fig. 2, right panel, was not a consistent finding. Scatchard analyses demonstrated only one class of P. vulgaris E-PHA receptor for each of the three cell suspensions.

Data from four such P. vulgaris E-PHA binding experiments (Table III) indicated a 1.8-fold increase in available binding sites on lymphocytes following oxidation (p < 0.05). Although cells that had been oxidized and reduced had an average of one-third less Con A sites than untreated cells, this was not statistically significant (p > 0.4). Association constant determinations were more variable, and the average 2-fold difference after oxidation and 1.7-fold decrease after oxidation plus reduction were not statistically different from untreated cells (p > 0.4).

In contrast to the results with mushroom PHA-B and P. vulgaris E-PHA, the binding of 125I-Con A to lymphocytes was slightly decreased after either oxidation, or oxidation plus reduction (Fig. 3 and Table III). The differences were small, however, and were not statistically significant (p > 0.4) due to greater variability in quantitative results. Affinity of Con A for the receptor site was similar in the three cell populations (p > 0.4). Scatchard analyses again demonstrated only one class of receptor.
Table III

Lectin binding to calf lymphocytes

Binding studies were performed as described under "Experimental Procedure" using "I-labeled mushroom PHA-B, Phaseolus vulgaris E-PHA, or Con A, and untreated, NaIO4-oxidized, or oxidized and NaBH4-reduced calf lymphocytes immediately after treatment. Means of five experiments (mushroom PHA-B) or four experiments (P. vulgaris E-PHA, Con A) are presented.

| Lectin           | Cell Treatment | Untreated | Oxidation and reduction |
|------------------|----------------|-----------|-------------------------|
| Mushroom PHA-B   | Sites          | 1.42      | 3.62*                   |
|                  |                | 3.59      | 8.54*                   |
|                  | Ks             | 0.94      | 20.0*                   |
| P. vulgaris E-PHA| Sites          | 2.17      | 3.97*                   |
|                  |                | 2.45      | 1.23                    |
|                  |                | 1.36      | 1.47                    |
| Con A            | Sites          | 7.32      | 5.79                    |
|                  |                | 0.15      | 0.16                    |

* Sites x 10^9/cell.
* 0.025 < p < 0.05 compared to untreated cells.
* 0.05 < p < 0.1.
* Ks association constant, x 10^9 M^-1.

Composition of Calf Lymphocyte Plasma Membranes—

Purified calf lymphocyte plasma membrane suspensions were oxidized using the same conditions as described for calf lymphocyte suspensions. To each 10 mg of membrane protein (Lowry) was added 5 ml of 3.7 x 10^-4 M NaIO4/0.005 M sodium phosphate, pH 7.4/0.15 M NaCl for 15 min at 25°C in light, and the membranes were then centrifuged and washed with the same buffer without NaIO4. The membranes were then hydrolyzed in conditions appropriate for the assay of amino acids or sugars as described under "Experimental Procedure." Sugar composition of the untreated and oxidized plasma membranes was similar except for sialic acid (Table IV). The thioarbituric acid assay revealed only 69% of the original activity in oxidized membranes. Since mild oxidation of N-acetylneuraminic acid yields a product NeuNAc-7-ald which reacts about half as well in the thioarbituric acid assay (21), the results suggested incomplete oxidation (about 60%) of sialic acid in lymphocyte plasma membranes to aldehydes. Contents of other sugars were not significantly different in oxidized membranes, and there was no loss of fucose content with oxidation.

Amino acid analysis showed no significant degradation of amino acids by oxidation (Table V). In particular, amino acids sensitive to non-Malapradian oxidation, threonine, serine, proline, cystine, and methionine, were unchanged within experimental error.

Sialic Acid Chromatography—Since the only apparent change in oxidized membranes was a decrease in thioarbituric acid-assayable material, chromatography was performed to more precisely determine quantitative and qualitative aspects of the oxidation. Normal membranes, oxidized membranes, and oxidized membranes reduced by NaBH4, were each hydrolyzed in dilute acid. The hydrolysate after centrifugation was subjected to descending paper chromatography in various solvents (Table VI). Migration of sugars was quantitated by
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**TABLE IV**

Calf lymphocyte plasma membrane composition

| Content                  | Molar Ratio | Oxidized, untreated | Oxidized, treated |
|--------------------------|-------------|---------------------|-------------------|
| µmol/mg                  |             | µmol/mg             | µmol/mg           |
| Sialic acids             |             | 0.48                | 0.03              |
| Mannoside                |             | 0.09                | 0.88              |
| Galactose                |             | 0.15                | 0.154             |
| Fucose                   |             | 0.06                | 0.073             |
| Glucosamine              |             | 0.27                | 0.277             |
| Galactosamine            |             | 0.06                | 0.064             |

**TABLE V**

Amino acid composition of calf lymphocyte plasma membranes

| Amino acid | Residues/1000 | Unreduced membranes | Oxidized membranes | Ratio |
|------------|---------------|---------------------|--------------------|-------|
| Aspartic   | 117           | 120                 | 1.03               |
| Threonine  | 60            | 65                  | 1.08               |
| Serine     | 112           | 112                 | 1.0                |
| Glutamine  | 131           | 135                 | 0.98               |
| Proline    | 61            | 66                  | 1.08               |
| Glycine    | 105           | 109                 | 1.04               |
| Alanine    | 83            | 89                  | 1.07               |
| Cystine    | 20            | 18                  | 0.9                |
| Valine     | 64            | 72                  | 1.12               |
| Methionine | 15            | 13                  | 0.87               |
| Isoleucine | 21            | 21                  | 1.00               |
| Leucine    | 82            | 70                  | 0.85               |
| Tyrosine   | 37            | 32                  | 0.96               |
| Phenylalanine| 71           | 78                  | 1.10               |

**TABLE VI**

Chromatographic behavior of sialic acids

| Substance applied | Mobility in solvent* |
|-------------------|----------------------|
|                   | I        | II       | III      |
| Standard N-acetylneuraminic acid (Sigma) | 1.0 | 1.0 | 1.0 |
| Synthetic NeuNAc-7-ald | 1.82 | 1.52 | 1.42 |
| Synthetic NeuNAc-7 | 1.82 | 1.51 | N.T. |
| Synthetic NeuNAc-7-ald (Blumenfeld)* | 1.70 | 1.48 | 1.39 |
| Standard N-glycolyneuraminic acid (Sigma) | 0.54 | 0.72 | 0.57 |
| Untreated membrane hydrolysate | 0.71, 0.99 | 0.67 | 0.62, 1.08 |
| Oxidized membrane hydrolysate | 0.71, 1.76 | 0.69 | 0.64, 1.42 |
| Oxidized and reduced membrane hydrolysate<sup>c</sup> | 0.54, 1.90 | N.T.<sup>c</sup> | 0.63 |

* Mobility relative to N-acetylneuraminic acid.
<sup>a</sup> Assayed by migration of tritium label.
<sup>c</sup> N.T., not tested.

**RESULTS**

Thiobarbituric acid stain (22) or liquid scintillation counting.

Standards used included N-acetylneuraminic acid and N-glycolyneuraminic acid; NeuNAc-7, and NeuNAc-7-ald. The latter two were synthesized as described by Liao et al. (21). In addition, NeuNAc-7 generously provided by Dr. O. O. Blumenfeld was included. The relative mobility of these standards in the solvent systems employed are shown in Table VI. The relative migration rates of N-acetylneuraminic acid and N-glycolyneuraminic acid agree with previously published results in Solvent II (93).

Hydrolase from untreated and oxidized plasma membranes each showed two pink spots after staining the chromatograms from Solvents I or III with thiobarbituric acid. Untreated plasma membrane hydrolases co-chromatographed with N-glycolyneuraminic acid and N-acetylneuraminic acid. Oxidized plasma membrane hydrolases co-chromatographed with N-glycolyneuraminic acid, NeuNAc-7-ald, and NeuNAc-7. Hydrolysates from reduced plasma membranes assayed by tritium migration co-chromatographed with N-glycolyneuraminic acid, accounting for 93% of the radioactivity, and with NeuNAc-7 and NeuNAc-7-ald, accounting for only 7% of the radioactivity. No oxidized or oxidized and reduced membrane hydrolase co-chromatographed with N-acetylneuraminic acid. Conversely, no untreated membrane hydrolase co-chromatographed with NeuNAc-7 or NeuNAc-7-ald.

**Neuraminidase Treatment of Lymphocytes—Calf lymphocytes were pretreated with Vibrio cholera neuraminidase (Table VII). This enzyme was active, as measured by its ability to remove 0.79 ± 0.1 µmol of sialic acid (measured as N-acetylneuraminic acid) from 10<sup>10</sup> calf lymphocytes (mean ± 1 S.E. for four experiments) or 0.63 µmol of sialic acid from 10<sup>10</sup> human erythrocytes (one experiment) in 90 min at 37°C.** Sonicated calf lymphocytes hydrolyzed in 0.05 M H<sub>2</sub>SO<sub>4</sub> at 80°C for 1 hour contained 1.2 µmol of free sialic acid per 10<sup>10</sup> cells. Thus, neuraminidase was able to remove 66% of the sialic acid. Lymphocytes which had been treated with neuraminidase prior to NaIO<sub>4</sub> oxidation exhibited slightly decreased rates of DNA synthesis compared to untreated cells. Neuraminidase treatment did not decrease the stimulation of DNA synthesis by P. vulgaris, indicating that its effect on NaIO<sub>4</sub>-oxidized cells was not a consequence of general inhibition of DNA synthesis. Thus, surface sialic acids were specifically required for the periodate-induced stimulation of DNA synthesis in calf lymphocytes.
Conformational changes in lectin receptors occurring only in temperature was chosen so that the study might also reflect the number of binding sites calculated from the binding studies underestimates the actual number of receptor sites. It is likely that the numbers of binding sites, altered affinity of the lectin for the cell surface glycoprotein receptor sites were studied processes in NaIO₄-transformed lymphocytes free of non-lymphoid proteins such as lectins or antigens which might coat the surface of responding lymphocytes; and (c) the stimulus for transformation, NaIO₄, need be present for only a short period of time to commit responding cells to increased DNA synthesis (24).

Lectin binding to available glycoprotein receptor sites was used as a probe for structural alterations in the surface of oxidized lymphocytes. Induction of heterogeneity in the binding sites, altered affinity of the lectin for the cell surface receptors, or changes in the number of receptors for the lectin each would reflect a structural change. The lectins studied were chosen because of their different glycopeptide specificities. P. vulgaris E-PHA binds to glycopeptides with N-glycosidically linked oligosaccharides containing galactose → N-acetylgalcosamine residues, especially if mannose is present in the "core" of the oligosaccharide chain (25). Mushroom PHA-B binds to glycopeptides with O-glycosidically linked galactose → N-acetylgalactosamine residues (7). Con A binds to glycopeptides containing mannose in an α configuration.

Because each of the lectins used is a multivalent molecule, it is likely that the numbers of binding sites calculated from the binding studies underestimate the actual number of receptor sites.

In limited studies of lectin binding at 4°, the amount of mushroom PHA-B bound to lymphocytes at 4° was only 37% of that at 25°, and the amount of P. vulgaris E-PHA bound at 4° was only 76% of that at 25°. Thus, lectin binding at 25°, used in all experiments reported under "Results," is probably greater than if similar experiments were performed at 0-4°, temperatures below the temperature-dependent phase transition proposed to occur in eukaryotic cell membranes (26). The higher temperature was chosen so that the study might also reflect conformational changes in lectin receptors occurring only in more fluid plasma membranes. Binding was not studied after NaBH₄ reduction of otherwise untreated lymphocytes since NaBH₄ reduction alone did not alter the rate of DNA synthesis.

Although the cell suspensions studied consisted nearly entirely of small and medium lymphocytes, subpopulations of lymphocytes (T cells, B cells, or others) were not evaluated. Scatchard analyses and double reciprocal plots of the binding data both indicated only one class of receptor for each lectin. This suggests that receptors on various subpopulations of cells were identical, although the possibility remains that different lymphocyte subpopulations had different numbers of receptors. It is unlikely that leakage of any intracellular molecules modified the lectin binding to intact cells in these experiments, since all cells were washed in medium prior to the binding assay, since nearly all cells were viable, and since the binding assays were performed immediately after oxidation or oxidation and reduction.

Results of the binding studies documented a significant increase in numbers of P. vulgaris and mushroom PHA-B receptor sites on oxidized lymphocytes. The oxidized receptors appeared to be homogeneous by Scatchard analyses suggesting that the additional receptor sites were glycoproteins with oligosaccharides similar in structure to those of unoxidized lymphocytes. This is compatible with a rearrangement in surface molecules permitting additional lectin to bind to glycoprotein receptors of identical or similar structure, and does not imply production of new receptor sites. New synthesis of additional receptor sites is also unlikely, because of the brief duration of NaIO₄ exposure prior to performing the binding study.

It is of interest that the apparent affinity of P. vulgaris E-PHA for nonoxidized and oxidized lymphocyte receptors was equal. This is consistent with observations on P. vulgaris E PHA receptors of human erythrocyte glycopeptides, which bind P. vulgaris E-PHA equally well before and after neuraminidase treatment (7). In contrast, the apparent affinity of mushroom PHA-B for oxidized calf lymphocyte receptors was increased compared to receptors on untreated cells. This conformational change in oxidized lymphocyte receptors may be related to oxidation of membrane sialic acids, since mushroom PHA-B has been shown to have a much greater affinity for erythrocyte glycopeptides after removal of sialic acid (7).

Con A binding to lymphocytes was not significantly changed by oxidation, or oxidation plus reduction, indicating that the changes in mushroom PHA-B and P. vulgaris E-PHA binding were related to specific membrane alterations. Possible explanations for the differences are: (a) Con A binds to a surface structure distinct from P. vulgaris E-PHA and mushroom PHA-B on calf lymphocytes; (b) undetected surface conformational changes are insufficient to permit more Con A molecules to bind; and (c) maximal amounts of Con A are bound even to oxidized lymphocytes. Further evidence that alterations of receptor sites by oxidation are specific is that NaBH₄ reduction of lymphocytes reduced the number of binding sites for both P. vulgaris E-PHA and mushroom PHA-B to levels equal to or below those of untreated cells, but did not change the binding characteristics of Con A.

Analysis of the composition of oxidized plasma membranes revealed only a change in sialic acids. This suggests that only mild Malapradian oxidation occurred under the conditions of periodate used in these experiments, and that more severe Malapradian oxidation of hexoses and hexosamines within the oligosaccharide chains of glycoproteins or glycolipids did not

### Table VII

| Treatment of lymphocytes | Oxidation | Neuraminidase | P. vulgaris E-PHA (32 µg/culture) | Stimulation index |
|--------------------------|-----------|---------------|----------------------------------|------------------|
|                          | -         | -             | +                                | 11.9             |
|                          | +         | -             | +                                | 18.9             |
|                          | -         | +             | +                                | 0.71             |
|                          | +         | +             | +                                | 0.47             |
|                          | -         | -             | +                                | 9.6              |
|                          | -         | -             | -                                | 1.0              |

### DISCUSSION

Stimulation of DNA synthesis by NaIO₄ treatment of lymphocytes is of particular interest because (a) the events mediating lymphocyte transformation and initiation of DNA synthesis are not known, and their identification would have important implications in immunology and oncology; (b) one can study processes in NaIO₄-transformed lymphocytes free of non-lymphoid proteins such as lectins or antigens which might modify the lectin binding to intact cells in these experiments, since all cells were washed in medium prior to the binding assay, since nearly all cells were viable, and since the binding assays were performed immediately after oxidation or oxidation and reduction.

Results of the binding studies documented a significant increase in numbers of P. vulgaris and mushroom PHA-B receptor sites on oxidized lymphocytes. The oxidized receptors appeared to be homogeneous by Scatchard analyses suggesting that the additional receptor sites were glycoproteins with oligosaccharides similar in structure to those of unoxidized lymphocytes. This is compatible with a rearrangement in surface molecules permitting additional lectin to bind to glycoprotein receptors of identical or similar structure, and does not imply production of new receptor sites. New synthesis of additional receptor sites is also unlikely, because of the brief duration of NaIO₄ exposure prior to performing the binding study.

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Analysis of the composition of oxidized plasma membranes revealed only a change in sialic acids. This suggests that only mild Malapradian oxidation occurred under the conditions of periodate used in these experiments, and that more severe Malapradian oxidation of hexoses and hexosamines within the oligosaccharide chains of glycoproteins or glycolipids did not
occurs. However, since analyses were performed on crude membrane preparations containing many different glycoproteins and glycolipids, the techniques used might not detect oxidation of hexose, hexosamine, or amino acid residues accounting for less than 10% of the total. In addition, these experiments did not study whether structural changes occurred in glycolipid fractions as well. However, it is unlikely that oxidation of glycolipids would result in altered binding of the lectins studied, since purified mammalian cell membrane receptors for these lectins are glycoproteins (7, 25, 27). The lack of major composition changes of oligosaccharide content of the oxidized plasma membranes in this study suggests, therefore, that the altered lectin binding reflects a conformational change in glycoproteins due to oxidation of sialic acids.

The nature of the oxidation of sialic acids was incompletely studied in the experiments reported. Although there is good evidence for complete conversion of N-acetylmuramic acid to NeuNac-7-ald by periodate in the calf lymphocytes (as in sialoglycopeptides and erythrocytes (21, 28, 29)), the result of N-glycolylnuraminic acid oxidation is not characterized, since the oxidized products of N-glycolylnuraminic acid co-chromatographed with unaltered N-glycolynuraminic acid. Studies are currently in progress to identify the oxidation products of N-glycolylnuraminic acid and of calf lymphocyte membranes by gas chromatography. This is important since 93% of the incorporation of NaIB3H, was into N-glycolylnuraminic acid derivatives and since N-glycolylnuraminic acid accounts for 53% (30) to 63% (31) of bovine fibrinogen or 93% of the incorporation of NaB3H, was into N-glycolylneuraminic acid derivatives and since N-glycolylneuraminic acid is not capable of initiating and sustaining lymphocyte DNA synthesis in lymphocytes by oxidation (3, 36). These data from a "resting" state to a "stimulated" or transformed state. It has been observed in other cell systems that cell-cell interaction is necessary for, or plays a major role in lymphocyte transformation (32, 33). However, in calf lymph node lymphocytes, neither direct cell-cell interaction nor soluble mediators are necessary for maximal lymphocyte transformation. These studies did not identify which lymphocyte subpopulation was transformed by oxidation.

A mechanism proposed for the mediation of growth, DNA synthesis, or morphologic transformation of cells, is that a change in plasma membrane conformation or composition in some way signals the control process within the cell to change from a "resting" state to a "stimulated" or transformed state. This hypothesis has been applied to fibroblast growth in tissue culture (34, 35), to transformation of lymphocytes by lectins and anti-immunoglobulin antibody, and to stimulation of DNA synthesis in lymphocytes by oxidation (3, 36). These data support this hypothesis. Experiments are in progress to identify the sialic acid-containing membrane glycoproteins whose oxidation is associated with lymphocyte transformation.  

References

1. Parker, J. W., O'Brien, R. L., Lukes, R. J., and Steiner, J. (1979) Lancet 1, 103-104  
2. Novogrodsky, A., and Katchalski, E. (1971) FEBS Lett. 12, 327-330  
3. Novogrodsky, A., and Katchalski, E. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1824-1827  
4. Novogrodsky, A. (1974) Eur. J. Immunol. 4, 466-468  
5. Novogrodsky, A., and Katchalski, E. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3207-3210  
6. Weber, T., Nordman, C. T., and Grasbeck, R. (1967) Scand. J. Hematol. 4, 77-80  
7. Presant, C. A., and Kornfeld, S. (1972) J. Biol. Chem. 247, 7087-7095  
8. Hunter, W. M. (1967) in Handbook of Experimental Immunology (Weir, D. M., ed.) pp. 608-654, Blackwell Publishing Co., London  
9. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 600-660  
10. Walter, C. (1974) J. Biol. Chem. 249, 699-703  
11. Steck, T. L., and Wallach, D. F. H. (1965) Biochem. Biophys. Acta 97, 510-522  
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275  
13. Bartlett, G. (1968) Methods Enzymol. 12B, 163-166  
14. Warren, L. (1979) J. Biol. Chem. 234, 1971-1975  
15. Kornfeld, R., Keller, J., Baenziger, J., and Kornfeld, S. (1971) J. Biol. Chem. 246, 3279-3286  
16. Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955) J. Biol. Chem. 217, 859-866  
17. Unsche, L., and Shettles, L. B. (1948) J. Biol. Chem. 175, 596-603  
18. Weaver, R. A., and Boyle, W. (1969) Biochim. Biophys. Acta 173, 377-388  
19. Kornfeld, R., and Siemers, C. (1974) J. Biol. Chem. 249, 1295-1301  
20. Earl, D. C., and Korner, A. (1965) Biochem. J. 94, 723-734  
21. Liao, T.-H., Gallop, P. M., and Blumenfeld, O. O. (1973) J. Biol. Chem. 248, 8247-8253  
22. Warren, L. (1970) Nature 186, 277  
23. Svennerholm, E., and Svennerholm, L. (1958) Nature 181, 1154-1155  
24. Parker, J. W., O'Brien, R. L., Steiner, J., and Paolilli, P. (1973) Cell. Res. 78, 279-286  
25. Kornfeld, R., and Kornfeld, S. (1970) J. Biol. Chem. 245, 2369-2374  
26. Noonan, K. D., and Burury, M. M. (1973) J. Biol Chem. 248, 4286-4292  
27. Allan, D., Auger, J., and Crumpion, M. J. (1972) Nature New Biol. 236, 93-95  
28. Blumenfeld, O. O., Gallop, P. M., and Liao, T. H. (1972) Biochem. Biophys. Res. Commun. 48, 242-251  
29. Van Benten, L., and Ashwell, G. (1971) J. Biol. Chem. 246, 1888-1894  
30. Chandrasokhar, N., Osbahr, A. J., and Laki, K. (1966) Biochem. Biophys. Res. Commun. 32, 577-576  
31. Karkas, J. D., and Chaffage, E. (1964) J. Biol. Chem. 239, 949-957  
32. O'Brien, R. L., Parker, J. W., Paolilli, P., and Steiner, J. (1974) J. Immunol. 112, 1884-1890  
33. Novogrodsky, A., and Gery, L. (1972) J. Immunol. 6, 1278-1281  
34. Burger, M. M., and Noonan, K. D. (1970) Nature 228, 512  
35. Svennerholm, E., and Burger, M. M. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3825-3827  
36. Zatz, M. M., Goldstein, A. L., Blumenfeld, O. O., and White, A. (1972) Nature New Biol. 240, 258-255