Extraction of Human Organ-specific Cancer Neoantigens from Cancer Cells and Plasma Membranes with 1-Butanol

Nabil Labateya, David M. P. Thomson, Margaret Durko, George Shenouda, Laura Robb, and Rosemarie Scanzano

Division of Clinical Immunology, Montreal General Hospital, Montreal, Quebec, Canada H3G 1A4

ABSTRACT

Immunoprotective tumor antigens of experimental tumors are selectively extracted by 1-butanol. Human organ-specific cancer neoantigens (OSNs) are tumor substances in cancer extracts to which patients with cancer of the same organ respond in the in vitro assay of leukocyte adherence inhibition. Here we determined whether OSNs as measured by leukocyte adherence inhibition assay are also selectively solubilized by 2.5% (v/v) 1-butanol. Butanol extracts of live tissue-cultured human cancer cells as well as extracts of primary breast cancer contained OSNs as determined by leukocyte reactivity in leukocyte adherence inhibition. With two-phase butanol, OSN activity was recovered in the aqueous and not in the organic phase, indicating that OSN is not a lipoprotein. The butanol-soluble OSN, whether allogeneic or autologous, was recognized by the T4 subset of T-cells in association with Class II major histocompatibility complex antigens of monocytes. Autologous OSN was extracted from membrane preparations of autologous primary cancer. Butanol extracts contained the previously identified M₄ 40,000 protein OSN. Butanol removed about 50% of the M₄ 40,000 protein OSN from live cancer cell membranes. Probably because of residual OSN in the membrane fragments and the ability of OSN to reassociate with the membrane, the T8 subset of pure T-cells responded positively to autologous cancer extracts. Passage of the autologous extract through an anti-Class I major histocompatibility complex antigen affinity column but not through a control affinity column negated the activity of the extract with pure autologous T-cells. The results indicate that human OSNs share with immunoprotective tumor antigens of experimental tumors the unique physicochemical property of being selectively extracted by 2.5% butanol.

INTRODUCTION

It is unknown whether human cancers possess immunoprotective tumor antigens similar to those of experimental tumors since many of the principles learned from experimental tumors cannot be applied to human cancers (1, 2). From a synthesis of research in laboratories worldwide, a picture emerges of human immunological reactivity to cancer. To date, three substances in tumors have been defined as initiating cellular immunological reactivity in the tumor host: MBP³ (3-5); T-antigen (6), the immediate precursor of the human blood group MN antigens; and OSN (7, 10). The in vitro assay of LAI (11), in addition to its use to detect immunological reactivity to defined antigens (12), bacteria, fungi, viruses, and animal tumors (9, 10), has been successfully applied to detect human tumor host sensitivity to MBP (13), T-antigen (14), and OSNs (9, 10). The LAI response of cancer patients to crude extracts of human tumor is organ specific (9, 10, 15, 16). With samples of blood (17-19), of tumor (20), and of tumor isolates (21) coded by impartial investigators, the same marked OSN specificity was observed. Nonadherence in LAI is induced in about 30% of bystander cells by mediators released from those cells that are sensitized to and bind antigen (22, 23). Pharmacological studies indicate that the final mediators are oxidative products of arachidonic acid, in particular, the chemoattractant leukotriene B₄ (24, 25). In congenic mice, the extent of chemoattractant-induced nonadherence strongly correlates with the inflammatory response in vivo and chemoattractant-induced macrophase migration in vitro (26). Recognition of antigens in LAI can be mediated by antibody-dependent monocytes (27, 28) or by T-cells (9, 10, 29-31).

Preimmunization of syngeneic experimental animals with irradiated, transplantable tumor cells, subcellular fractions, or soluble extracts engenders a specific immune response that protects the animals from a subsequent challenge with the same tumor. LeGrue et al. (32) discovered that single-phase 1-butanol extraction of viable tumor cells selectively releases immunogenic tumor antigens (33-36). Other investigators have confirmed that one-phase butanol solubilizes unique and common immunoprotective antigens from murine colon tumor cells (37), common immunoprotective antigens from murine melanoma cells (34, 38), and common or “tissue-specific” immunoprotective antigens from rat colon tumors which are absent from normal colon, kidney tumor, or lymphoma (39, 40). Butanol-soluble tumor antigens from murine mammary tumors also stimulate specific LAI responses which correlate with tumor growth (41). We decided to determine whether OSNs of human cancer are selectively extracted by one-phase butanol. The tumor antigen activity of the extracts was tested with leukocytes from tumor hosts by the well-characterized in vitro assay of leukocyte adherence inhibition (9, 10).

Here we show that OSN activity is solubilized by one-phase butanol extraction of either live cancer cells or cellular debris. Butanol-soluble OSN from allogeneic breast cancer was recognized by the T4 subset of human T-cells in association with Class II MHC determinants of monocytes. When the OSN was prepared from autologous tumor extracts the T8 subset also responded probably because of residual OSN in the membrane fragments or reassociation with the membrane fragments. Human OSNs and immunoprotective experimental tumor antigens share the same physicochemical property of being selectively solubilized by 2.5% 1-butanol.

MATERIALS AND METHODS

Tumor Cell Lines. The human cell lines grown and used for butanol extraction were NCI-H69 (lung cancer), HCT-15 (colon cancer), T-24 (bladder cancer), and HU4459 (breast cancer). The cells were grown adherent to plastic or glass roller culture bottles in RPMI (Gibco) containing 0.013 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer (Boehringer Mannheim, Montreal, Canada), 0.2% sodium bicarbonate (Fisher Scientific, Montreal, Canada), and antibiotics as
previously described (42, 43). Cultures of spent medium or shed cells were negative for Mycoplasma; a Mycoplasma kit for staining Mycoplasma gave negative results (Hoechst); and polyclonal anti-Mycoplasma polyclonal antibody (MA Products; No. 30-965N) showed positive binding to complement-fixing Mycoplasma antigen but not to butanol extracts of cancer cells by solid-phase ELISA. MAb anti-p40 did not bind complement-fixing Mycoplasma antigen.

Butanol Extraction. Extraction was performed by 5-min incubation of confluent cells in the roller bottles with butanol in PBS containing 2.5% (v/v) 1-butanol without protease inhibitors. The butanol was collected, and the cells were washed twice with PBS before returning them to the growth medium. The cells grew vigorously after the weekly extractions. The resulting extract and PBS washes were pooled and centrifuged at 20,000 × g for 20 min. Residual butanol was removed by dialysis against three changes of 10 liters of PBS at 4°C over 24 h.

The butanol extract was clarified by ultracentrifugation at 160,000 × g for 1 h and then concentrated in an Amicon-stirred cell with a PM10 membrane. The yield from the butanol extract of the cells in one roller bottle (1585 cm² surface area) was about 10 mg protein. Protein was determined by the method of Lowry et al. (44) with crystalline bovine serum albumin as standard.

Cellular debris in spent medium from the tissue-cultured cells was collected by centrifugation at 8000 × g for 20 min, washed once in PBS, and stored at −40°C. When sufficient cellular debris had been collected, it was thawed and suspended at a ratio of 1 to 10 in 2.5% (v/v) 1-butanol in PBS containing 10 μg colchicine/ml (35) for about 10 min at room temperature. The insoluble material was removed by centrifugation at 20,000 × g for 20 min. The supernatant was dialyzed at 4°C against three changes of 20 liters of PBS, ultracentrifuged at 160,000 × g for 1 h, and then concentrated by ultrafiltration. The protein concentration of the extract was determined before storage at −40°C. Crude extracts of primary breast cancer were prepared and homogenized in PBS as previously described (10, 19). Half of the homogenate was then brought to 2.5% (v/v) 1-butanol in PBS for about 10 min at room temperature. The butanol and untreated extract of primary breast cancer were then processed as described above for the cellular debris.

To determine whether the OSN was a proteolipid, the cellular debris was extracted with 43% (v/v) butanol in PBS, giving a 2-phase system. The proteolipids are defined as polypeptides which, in a water/organic solvent 2-phase system, will partition into the organic phase (45, 46). After separation into a 2-phase system by centrifugation at 27,000 × g for 5 min, the lower aqueous phase was collected and treated as described above. The organic phase was evaporated under N₂ and reconstituted in 100% ice-cold ether. The ether was removed, and the precipitated protein was reconstituted with PBS. Most of the precipitate resuspended, and the clarified PBS contained no measurable protein.

Leukocytes. Heparinized venous blood was obtained from untreated cancer patients and patients with benign disease. All patients with cancer were tested before surgery and confirmation of the diagnosis. Buffy coat PBL, mononuclear cells, and T-cells were prepared as previously described (25, 29, 30). The purity of T-cells was verified by flow cytometry on a FACS analyzer instrument as described previously (29, 30).

Antisera and Affinity Columns. Anti-T8 (Leu-2a), anti-T4 (Leu-3a), and anti-T3 (Leu-4) were purchased from Becton-Dickinson & Co., Mississauga, Ont. They were used at a dilution of 1:250 [1 μg anti-Leu-2a and anti-Leu-3a and 0.5 μg for anti-Leu-4], a concentration which was equipotent in coating most of the antigen on 1 × 10⁶ T-cells (31)]. Anti-Class I MHC (HLA-A, B, C) and anti-Class II MHC (HLA-DR) were purchased from Cappel Laboratories (Cochraneville, PA) and were used at 1:12 (0.8 μg) and 1:35 (0.3 μg) dilutions, respectively (13).

Anti-p40 OSN is directed to a cross-reactive framework determinant of lung and colon cancer cell surface molecules with a molecular weight of 40,000, which also possess separate OSN determinants (42, 47). Control MAb was from a hybridoma producing IgG to an irrelevant determinant. Anti-p40 OSN and control MAb were purified from either supernatant or ascites (47). MAb to Class I MHC determinant (W6/32) was purified from a producing hybridoma cell line (American Type Culture Collection) (47). The purified MAbs of both W6/32 and control IgG were linked separately to glutaraldehyde-activatedaryl hydroxylase-Sepharose 4B.

One ml of each affinity-linked antibody (29 mg/ml) was placed in a small, narrow column. The butanol extracts were divided into equal portions and slowly passed through the anti-Class I MHC antigen and control IgG columns. The bound material was recovered by elution with 0.5 M diethylamine (pH 11.5) and immediately neutralized with 1 M Tris-HCl (pH 7.8). The unbound and bound materials were dialyzed against PBS at 4°C and then concentrated by ultrafiltration. The unbound and bound materials were tested in LAI at the same protein concentration as the butanol extract applied to the affinity column.

Membrane Staining of Cancer Cells with MAb. NCI-H69 and HCT-15 cells were harvested from tissue culture flasks. Half of the cells were extracted with 2.5% butanol as described above. The butanol-extracted and unextracted cells were distributed into tubes and incubated with Hematall, an isotonic diluent [0.15 M NaCl/0.03 M KC1/0.15 M phosphate buffer (pH 7.5)] (Fisher Scientific, Montreal, Canada) with medium alone, with control MAb IgG, W6/32, and with MAb anti-p40. After 1 h the cells were washed with cold Hematall and then incubated with a fluorescein-labeled affinity-purified goat anti-mouse IgG antibody. The cells were washed and left in paraformaldehyde in Hematall and analyzed for membrane staining by flow cytometry on a FACS analyzer instrument.

A peroxidase-antiperoxidase assay with a Histogen peroxidase-antiperoxidase immunohistology immunoperoxidase staining kit (Genex Laboratories, Dublin, CA) was performed on harvested NCI-H69 and HCT-15 cells that were fixed to glass slides. The p40 OSN and Class I MHC molecules were detected by MAb anti-p40 and MAb W6/32, respectively, and background staining was detected by a control IgG MAb. The harvested cells, half were extracted with 2.5% butanol. The untreated and treated cells were smeared on slides, air dried, and fixed with acetone. Primary antibody from supernatants was added at a 1:5 dilution and was incubated for 1 h at 20°C. The slides were washed with PBS, incubated with link antibody, and then with labeling antibody (a soluble peroxidase-antiperoxidase complex). The entire antigen-antibody-enzyme complex was made visible by addition of a chromogenic substrate. In a reaction catalyzed by hydrogen peroxide, the substrate (aminoethylcarbazole) forms a red to brownish-red stain at the site of antigen localization on the cells. The slides were coded and scored as 0 to 4+ reactions.

LAI Activity. The LAI activity of PBL was tested on crude (particulate) cancer extracts as previously described by Grosser and Thomson (48). Organ-specific activity of the particulate cancer extracts or of butanol extracts of primary breast cancer was tested as previously described (48). The butanol extracts of tissue-cultured cells or tissue-cultured cellular debris were tested with a slightly modified assay (42, 47). In brief, 100 μg in 100 μl of an active control cancer extract, such as pancreatic cancer, were added to both sets of Tubes A and B. To A tubes were then added 10 μl of the butanol extract. Medium 199, 300 μl, and 1 × 10⁴ leukocytes in 100 μl were added to both sets of tubes as in the standard LAI assay. The final volumes in Tubes A and B were 510 and 500 μl, respectively. Since the control extract used in both tubes was an active cancer extract, it was not of the same organ as that of the leukocyte donor or that of the butanol extract.

The use of 100 μg of a crude cancer extract results in both maximum peak LAI response and an optimum background number of nonadherent cells for accurate counting. Fetal calf serum at 1% can be substituted for the crude cancer extracts and induces a similar background of nonadherent cells. When the assay is conducted in the complete absence of proteins, the leukocytes adhere more firmly to the glass so that fewer leukocytes become nonadherent to either the mediators generated by the antigen-binding leukocytes or to even authentic chemoattractants (22).

The results were expressed as a NAI

\[
NAI = \frac{A - B}{B} \times 100
\]

where \( A \) is the number of cells nonadherent in a sample from tubes containing the crude butanol extract and crude control cancer extract, and \( B \) is the number of cells nonadherent in a sample from tubes containing the crude extract of the cancer cell line.
containing only the crude control cancer extract.

For crude cancer extracts NAI's > 30 are positive, since > 95% of control subjects have values < 30, and about 80% of patients with cancer have NAI's > 30 to an extract of cancer from the same organ (9, 10).

RESULTS

Bell-shaped LAI Response to Butanol Extracts of Tissue-cultured Materials. The butanol extracts were always titrated before wide-scale testing to determine the amount that consistently gave a positive LAI response. Table 1 shows a typical antigen concentration-response curve to a butanol extract from live tissue-cultured lung cancer cells. The antigen concentration-response curves were characterized by a peak response at about 1 to 2 µg, and by a 75% decrease in the magnitude of LAI relative to the peak at about 15 µg. LAI activity was often detected with lower concentrations such as 0.5 to 0.75 µg, but individual responses were lower as were the number of positive responses. The antigen concentration-response curve for leukocytes from patients with benign disease was flat and low, and no response was elicited with higher or lower antigen concentrations than those shown in Table 1. False-positive results averaged about 8% and were considered to be technical false-positives, since repeat tests were negative and were as frequent when no butanol extract was added.

Organ Specificity of Butanol Extracts. LAI responses to butanol extracts prepared from tissue-cultured cells of colon, breast, bladder, and lung cancer were evaluated. Organ-specific LAI responses were observed to the butanol extracts. Table 2 shows fairly typical results obtained with the lung cancer extract. Of lung cancer patients, about 80% reacted positively to the NCI-H69 lung cancer butanol extract. Technical false-positives were less than 8% as determined by the positive responses to the blank control. Of patients with other cancers or benign diseases, positive responses to the lung butanol extract were few and no greater than the technical false-positive rate (Table 2). Partial cross-reactive responses were observed with leukocytes from breast cancer patients and had been reported previously (19, 20, 42, 49, 50). When we evaluated LAI responses in more detail by testing patients with either breast or lung cancer at the same time to both particulate and butanol extracts of both cancers, lung cancer patients responded less than 50% as well to breast cancer extracts as compared to the lung cancer extracts, either particulate or butanol (P < 0.001). Leukocytes from patients with breast cancer exhibited a similar pattern in that the response to particulate and to butanol breast cancer extracts was almost twice as great as the LAI response to the comparable lung preparation (P < 0.001). The results suggest that the OSNs in extracts of breast and lung are similar but not identical.

Cellular debris was collected from spent medium of the NCI-H69 lung cancer cell line, was extracted with butanol, and was found to have organ-specific activity when tested in LAI (Table 3). The antigen concentration-response curves were bell shaped and often shifted to the right as higher concentrations of antigen (5 to 10 µg) were required to stimulate peak responses. Similar results were obtained for butanol extracts of cellular debris from other cell lines (not shown). To determine whether the OSN was a lipoprotein, the cellular debris was extracted with 43% (v/v) butanol which gave a 2-phase system. OSN activity was recovered in the aqueous phase, whereas the organic phase contained no LAI activity (Table 3).

T-Cell Response to Butanol Extracts of Primary Breast Cancer. Previously, we showed pure T-cells from breast cancer patients to give positive LAIs only to extracts of particulate autologous breast cancer (29–31). In the initial series of experiments, we determined the cell mechanism responsible for responding to the OSN when particulate extracts of primary breast cancer were treated with 2.5% butanol. PBL from breast cancer patients gave positive responses to the particulate (100 µg/assay) or butanol extracts (100 µg/assay) of allogeneic and autologous primary breast cancer (Table 4). PBL from control subjects gave no positive responses. Positive responses of pure T-cells from breast cancer patients were limited to the butanol extracts of autologous breast cancer (Table 4). T-cells from control subjects gave no positive responses (Table 4).

Effect of Removing Class I MHC Antigens from Target Tissues. The T8 subset of T-cells usually recognizes foreign antigens in association with Class I MHC antigens on the target cell surface (19). However, we showed that the T-cell response to the OSN was not dependent on class I MHC antigens (Table 5).

### Table 1: Titration of butanol extract for LAI activity

<table>
<thead>
<tr>
<th>Concentration of butanol extract (µg/assay tube)</th>
<th>NAI to butanol extract of lung cancer cells (NCI-H69)</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.0</td>
<td>18 ± 9*</td>
<td>1 ± 8</td>
</tr>
<tr>
<td>10.0</td>
<td>34 ± 14</td>
<td>2 ± 11</td>
</tr>
<tr>
<td>5.0</td>
<td>52 ± 12</td>
<td>3 ± 8</td>
</tr>
<tr>
<td>2.0</td>
<td>71 ± 16</td>
<td>5 ± 10</td>
</tr>
<tr>
<td>1.0</td>
<td>68 ± 13</td>
<td>1 ± 4</td>
</tr>
<tr>
<td>0.75</td>
<td>38 ± 1</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>0.50</td>
<td>32 ± 8</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>0.25</td>
<td>27 ± 9</td>
<td>1 ± 7</td>
</tr>
<tr>
<td>0.10</td>
<td>13 ± 5</td>
<td>9 ± 15</td>
</tr>
<tr>
<td>0.00</td>
<td>8 ± 10</td>
<td>-1 ± 3</td>
</tr>
</tbody>
</table>

*Mean ± SE.

### Table 2: Leukocyte adherence inhibition to butanol extract of tissue-cultured lung cancer cells (NCI-H69)

<table>
<thead>
<tr>
<th>Leukocyte donor</th>
<th>NAI to particulate cancer extract*</th>
<th>NAI to coded extracts of butanol extract of lung cancer cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td>75 ± 8*</td>
<td>5 ± 3 (56, 62 ± 12* (84))</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>61 ± 11</td>
<td>2 ± 7 (0)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>67 ± 8</td>
<td>-2 ± 2 (0)</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>65 ± 15</td>
<td>8 ± 15 (0)</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>45 ± 2</td>
<td>4 ± 6 (0)</td>
</tr>
<tr>
<td>Benign diseases</td>
<td>11 ± 4</td>
<td>-2 ± 2 (0)</td>
</tr>
</tbody>
</table>

*Particulate extract was the same as the diagnosis of the donor except for patients with benign disease when it was lung cancer.

*Mean ± SE.

*Numbers in parentheses, percentage positive.

*Significantly different from groups without lung cancer (excluding breast) at P < 0.001 as determined by Student's t-test.

*Significantly different from group with lung cancer at P < 0.05.
involved in antigen recognition can be determined, and the manner of MHC restriction can be inferred from experiments in which the T-cell differentiation antigens are coated with MAb before the antigen-induced response (13, 29–31, 53, 54). In addition, MAb to the T3 complex interferes with antigen-induced signal transduction in T-cells (53, 54). Accordingly, experiments were conducted to determine whether coating T8, T4, and T3 differentiation antigens with MAb affected the LAI response of either pure T-cells or mononuclear cells from breast cancer patients. The MAbS were used at concentrations that were not above the saturating dose for the markers, so that any antagonistic effects would not be suspect because of nonspecific or toxic effects of excess antibody. The response of pure T-cells was antagonized by anti-T8 but not by anti-T4. The effect of anti-T8 was dose dependent: 1.0 µg/ml/10⁷ T-cells inhibited LAI by 76%; 0.7 µg inhibited LAI by 66%; and 0.5 µg inhibited LAI by 24%. Anti-T3 also inhibited the pure T-cell response (Table 6) and was dose dependent: 0.5 µg inhibited by 77%; 0.35 µg inhibited by 58%; 0.25 µg inhibited by 31%; and 0.125 µg did not inhibit. The results indicate that the autologous tumor antigen in the butanol extract of primary breast cancer is recognized by the T8 subset of T-cells which are usually Class I MHC restricted.

Unlike autologous pure T-cells, the response of autologous mononuclear cells to butanol extracts of autologous primary breast cancer was not antagonized by anti-T8 (Table 6). Anti-T3 inhibited the response of autologous mononuclear cells by 46%, but anti-T4 did not inhibit. The results indicated that autologous mononuclear cells, besides the T8 recognition mechanism, had other cellular recognition mechanisms such as possibly antibody-dependent monocytes or certain T-cells because of the partial inhibitory effect of anti-T3. To exclude the T8 subset response to autologous tumor antigen and to eliminate antibody-dependent monocyte responses to OSN on membrane fragments in the butanol extract of primary cancer, as discussed later, experiments on mononuclear cell responses were undertaken with butanol extracts prepared from allogeneic tissue.

cell membrane, whereas the T4 subset recognizes soluble antigen in association with Class II MHC antigens on APC such as monocytes (51–53). To confirm that Class I MHC antigens were responsible for the pure T-cell response to autologous OSN in the butanol extracts, Class I MHC antigens were removed from the extracts by affinity chromatography. Pure T-cells gave positive responses to the butanol extract of autologous cancer before and after passage through the normal IgG affinity column (Table 5). In contrast, pure T-cells did not give positive responses to the butanol extract of autologous cancer passed through the anti-Class I MHC antigen affinity column. The material bound and eluted from either column possessed no LAI activity for pure T-cells. Mononuclear cells from breast cancer patients were also tested with the same materials. The unbound material from both the control IgG affinity column and the anti-Class I MHC affinity column elicited positive responses (Table 5). That from the anti-Class I MHC affinity column gave a diminished response, suggesting that OSN activity was removed along with the Class I MHC antigens. The results indicated that removal of Class I MHC antigens from the autologous butanol extract prevented pure T-cells from responding in LAI to the OSN but did not interfere with other cellular response mechanisms that might be responsible for OSN recognition, such as antibody-dependent monocytes or T4 subsets in association with monocytes.

Cell Phenotype Responding to Butanol-extracted OSN from Either Allogeneic or Autologous Cancer. The T-cell phenotype involved in antigen recognition can be determined, and the manner of MHC restriction can be inferred from experiments in which the T-cell differentiation antigens are coated with MAb before the antigen-induced response (13, 29–31, 53, 54). In addition, MAb to the T3 complex interferes with antigen-induced signal transduction in T-cells (53, 54). Accordingly, experiments were conducted to determine whether coating T8, T4, and T3 differentiation antigens with MAb affected the LAI response of either pure T-cells or mononuclear cells from breast cancer patients. The MAbS were used at concentrations that were not above the saturating dose for the markers, so that any antagonistic effects would not be suspect because of nonspecific or toxic effects of excess antibody. The response of pure T-cells was antagonized by anti-T8 but not by anti-T4. The effect of anti-T8 was dose dependent: 1.0 µg/ml/10⁷ T-cells inhibited LAI by 76%; 0.7 µg inhibited LAI by 66%; and 0.5 µg inhibited LAI by 24%. Anti-T3 also inhibited the pure T-cell response (Table 6) and was dose dependent: 0.5 µg inhibited by 77%; 0.35 µg inhibited by 58%; 0.25 µg inhibited by 31%; and 0.125 µg did not inhibit. The results indicate that the autologous tumor antigen in the butanol extract of primary breast cancer is recognized by the T8 subset of T-cells which are usually Class I MHC restricted.

Unlike autologous pure T-cells, the response of autologous mononuclear cells to butanol extracts of autologous primary breast cancer was not antagonized by anti-T8 (Table 6). Anti-T3 inhibited the response of autologous mononuclear cells by 46%, but anti-T4 did not inhibit. The results indicated that autologous mononuclear cells, besides the T8 recognition mechanism, had other cellular recognition mechanisms such as possibly antibody-dependent monocytes or certain T-cells because of the partial inhibitory effect of anti-T3. To exclude the T8 subset response to autologous tumor antigen and to eliminate antibody-dependent monocyte responses to OSN on membrane fragments in the butanol extract of primary cancer, as discussed later, experiments on mononuclear cell responses were undertaken with butanol extracts prepared from allogeneic tissue.

Table 4 Pure T-cell response to particulate or butanol extracts of primary breast cancer

<table>
<thead>
<tr>
<th>Leukocyte donor</th>
<th>Cell type tested</th>
<th>Type of primary breast cancer extract</th>
<th>NAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer (5)</td>
<td>PBL</td>
<td>Autologous particulate</td>
<td>56 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butanol</td>
<td>64 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allogeneic particulate</td>
<td>60 ± 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butanol</td>
<td>54 ± 9</td>
</tr>
<tr>
<td>Control subjects (5)</td>
<td>PBL</td>
<td>Autologous particulate</td>
<td>3 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butanol</td>
<td>-1 ± 6</td>
</tr>
<tr>
<td>Breast cancer (5)</td>
<td>T-cells</td>
<td>Allogeneous butanol</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autologous particulate</td>
<td>46 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butanol</td>
<td>47 ± 7</td>
</tr>
<tr>
<td>Control subjects (4)</td>
<td>T-cells</td>
<td>Allogeneous butanol</td>
<td>2 ± 8</td>
</tr>
</tbody>
</table>

* Particulate and butanol extracts of primary breast cancer gave peak responses at about 100 µg/exposure.

* Numbers in parentheses, number of subjects tested, each was always tested in duplicate, sometimes in triplicate.

* Mean ± SE.

* Significantly different from the control subjects at P < 0.02 as determined by Student's t test.

* Significantly different from autologous extract at P < 0.02 as determined by Student's t test.

Table 5 Effect on LAI response of T-cells and mononuclear cells of passage of butanol extract of autologous breast cancer through anti-Class I MHC affinity column

<table>
<thead>
<tr>
<th>Type of cell from breast cancer patient</th>
<th>NAI to butanol extract of autologous breast cancer</th>
<th>Anti-Class I MHC</th>
<th>Control IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cells (5)</td>
<td>55 ± 9</td>
<td>-1 ± 6</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>Mononuclear cells (5)</td>
<td>57 ± 12</td>
<td>41 ± 3</td>
<td>15 ± 8</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of subjects tested; each was always tested in duplicate, sometimes in triplicate.

* Mean ± SE.

* Significantly different from the untreated extract at P < 0.001.

* Significantly different from the unbound control affinity column at P < 0.005.

* Significantly different from the untreated extract at P < 0.01.

* Significantly different from the unbound control affinity column at P < 0.01.

Table 6 Effect of antibody to T-cell differentiation antigens T8, T4, and T3 and to Class I and II MHC antigens on LAI response of pure T-cells and mononuclear cells from breast cancer patients to autologous and allogeneic breast cancer extracts

<table>
<thead>
<tr>
<th>Effector leukocytes</th>
<th>MAb used to coat effector cells</th>
<th>NAI to breast cancer extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>Particulate</td>
<td>Butanol Particulate</td>
</tr>
<tr>
<td>Pure T-cells; 5 subjects tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>62 ± 5</td>
<td>61 ± 9</td>
</tr>
<tr>
<td>Anti-T8</td>
<td>15 ± 7</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Anti-T4</td>
<td>56 ± 10</td>
<td>65 ± 18</td>
</tr>
<tr>
<td>Anti-T3</td>
<td>14 ± 11*</td>
<td>14 ± 4*</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 subjects tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>59 ± 6</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>Anti-T8</td>
<td>81 ± 12</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>Anti-T4</td>
<td>74 ± 14</td>
<td>3 ± 6</td>
</tr>
<tr>
<td>Anti-T3</td>
<td>32 ± 7*</td>
<td>7 ± 5*</td>
</tr>
<tr>
<td>6 subjects tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>101 ± 17</td>
<td>95 ± 27</td>
</tr>
<tr>
<td>Anti-Class I MHC</td>
<td>70 ± 9</td>
<td>60 ± 11</td>
</tr>
<tr>
<td>Anti-Class II MHC</td>
<td>-3 ± 2*</td>
<td>72 ± 14</td>
</tr>
</tbody>
</table>

* Cells (1 × 10⁶/ml) were incubated in 1 ml medium containing diluted MAb anti-T8 (1.0 µg) - T4 (1.0 µg) - T3 (0.5 µg), diluted anti-Class I MHC (0.8 µg), or diluted anti-Class II MHC (0.3 µg) for 30 min at 4°C; washed once in Medium 199 to remove excess MAb; resuspended at 1 × 10⁷ cells/ml in Medium 199; and then plated at 1 × 10⁶ cells/0.1 ml in the LAI tubes.

* Mean ± SE.

* Each subject was always tested in duplicate, sometimes in triplicate.

* Significantly different from untreated cells at P < 0.001.

* Significantly different from untreated cells at P < 0.01.

* Significantly different from untreated cells at P < 0.05 as determined by Student's t test.
BUTANOL-EXTRACTED HUMAN OSN

Table 7 Effect of 2.5% butanol extraction of live tissue-cultured cancer cells on expression of cell surface p40 as measured by flow cytometry

<table>
<thead>
<tr>
<th>Reagent*</th>
<th>Expression of p40 molecule on cell surface of tissue-cultured cancer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematall</td>
<td>0.4 Before, 0.2 After</td>
</tr>
<tr>
<td>Control IgG</td>
<td>0.5 Before, 0.5 (0) After</td>
</tr>
<tr>
<td>Anti-p40</td>
<td>56 Before, 11 (80) After</td>
</tr>
<tr>
<td>Anti-Class I MHC</td>
<td>38 Before, 39 (0) After</td>
</tr>
</tbody>
</table>

* Cells were incubated for 1 h with primary MAb and then for 30 min with fluorescein-labeled goat anti-mouse IgG antibody. Control IgG, neat supernatant; anti-p40, neat supernatant; anti-Class I MHC, ascites diluted 1:10.

† Cells were shaken loose from roller bottles, and half were incubated for 5 min with 2.5% (v/v) 1-butanol and then washed in PBS.

Numbers in parentheses, percentage of decrease.

The experiments described in this paper indicate that butanol selectively extracts from live tissue-cultured cancer cell substances, as measured by antigen-induced LAI, that triggered LAI responses for cancer patients but not for subjects without cancer. Moreover, the response of leukocytes from cancer patients was limited to a butanol extract identical to the donor’s cancer, indicating that the response was to OSN. Cross-reactivity to a common tumor autoantigen, such as MBP or T, was not found. The magnitude of the LAI response plotted as a function of antigen concentration yielded a bell-shaped concentration-response relationship with a single optimal value for maximum LAI.

A completely soluble preparation of the OSN was obtained by butanol extracting live cancer cells. To such preparations, T4 helper cells in association with monocytes were responsible for mediating the LAI response. Antibody-dependent monocytes did not seem to react. When OSN was solubilized from the membranes of primary breast cancer, butanol either did not completely solubilize the OSN or the solubilized OSN reassociated with the membranes fragments. In this instance, T8 cytotoxic T-cells, T4 helper T-cells in association with monocytes, and antibody-dependent monocytes responded to particular and soluble forms of OSN.

Lamb et al. (55) report that antigen-specific human T-cell clones specific for defined peptides of Influenza A hemagglutinin are rendered unresponsive by incubation with moderately high concentrations of antigen. Matis et al. (56) showed that the magnitude of the proliferative response of specific T-cell clones is a function of the product of antigen concentration and the number of Class II molecules expressed on APC. They envisioned two mechanisms to account for the observed concentration-response relationship (56). (a) Maximal T-cell responses would result when a given number of cell surface receptors are bound by antigen-Class II molecular complexes, and relative inhibition of T-cell biochemical changes occurs as additional T-cell receptors are engaged by the antigen-Class II molecular complexes on the macrophage cell surface. (b) An alternative explanation for the diminished T-cell responses by high antigen concentrations derives from the observation that such antigen-activated T-cells produce a variety of lymphokines, and enhanced production of lymphokines could have a potential inhibitory effect (56). Antigen-induced LAI is mediated by leukotrienes (13, 24, 25, 30), and authentic leukotrienes produce similar bell-shaped nonadherence response curves (22). The observed decreased magnitude of LAI with high concentrations of antigen could result from enhanced production of leukotrienes to levels that have an anti-nonadherence effect. The critical role of both antigen concentrations and the number of Class II molecules on APC in determining proliferative responses (56) probably also explains why we failed to observe positive LAI responses to soluble OSN, or previously to MBP (13) when pure T-cells were reconstituted with autologous monocytes.

MBP and T-antigen stimulate LAI responses in patients with cancer (13, 14) and also have bell-shaped antigen concentration-response curves (13). MBP and T-antigen act as common autoantigens and lack organ specificity. The cross-reactivity between breast and lung cancer could exist because the extracts contained common autoantigens. If so, patients with cancers other than breast or lung should have shown cross-reactive LAI responses to the lung or breast cancer extracts which they did not. The partial cross-reactivity between breast and lung cancer was observed previously with particulate extracts (19, 20, 42, 49, 50) and purified p40 lung cancer OSN (42, 43, 47), and it...
most likely exists because of structural similarity between the antigenic determinants of the two OSNs. Antibody-dependent monocytes respond in LAI when particulate extracts of allogeneic or autologous cancer are used (13, 27, 28); Class I-restricted T-cells of the T8 subset respond in LAI when particulate extracts of autologous cancer are used (30, 31); and the Class II-restricted T4 subset of T-cells responds in LAI when soluble antigens (13) or purified p40 OSN4 are used and monocytes are also present. In all instances, the cells recognizing and binding antigen release leukotriene mediators that inhibit the glass adherence of about 30% of bystander cells (22–24). In this study, the T8 subset recognized autologous OSN and was restricted by Class I MHC antigens on the target tissues. The butanol autologous OSN was prepared from a particulate extract of primary breast cancer. We have expected butanol to completely solubilize the OSN. However, butanol removed only about 50% of p40 molecules from the membranes of live cancer cells. In addition, butanol-soluble molecules have the ability to reassociate with membranes (57, 58), and in the primary cancer extracts the membranes were not separated from the butanol-soluble material. Consequently, the Class I-restricted T-cells responded to OSN either not extracted or reassociated with membrane fragments containing Class I MHC molecules.

Since mononuclear cells from breast cancer patients responded to autologous as well as to butanol extracts of allogeneic breast cancer, a recognition mechanism distinct from that of the T8 subset existed. The butanol extracts from live cancer cells consisted of principally soluble material. The response to soluble allogeneic OSN by mononuclear cells was antagonized in a dose-dependent fashion by anti-T4, anti-T3, and -Class II MHC antigen, suggesting that the T4 (helper) subset was activated by antigen-Class II MHC molecule complexes on the surface of monocytes. The response of mononuclear cells to OSN associated with the tumor cell membrane was not inhibited by MAB to T-cell differentiation antigens or to Class II MHC antigens; the latter result is consistent with previous observations that antibody-dependent monocytes mediate responses to the OSN when it is allogeneic and associated with the tumor cell membrane (27, 28).

Soluble antigens require about 1 h for processing and presentation on the surface of the APC (59). The T4 subset prefers partially digested antigens (60–62); however, Class II-restricted T-cells are probably capable of recognizing some antigens that are not processed. Since the OSN in butanol extracts is soluble, it can immediately bind either intact or after processing to Class II MHC antigens of monocytes. By contrast, the OSN in particulate extracts is still part of cancer cell membrane fragments and cannot immediately bind to Class II molecules of APC, and the short assay time probably limits the amount of OSN solubilized from the membrane by phagocytic cells. The failure to observe an antibody-dependent monocyte response to butanol-soluble OSN suggested that anti-tumor IgG on the monocytes cell surface is to a univalent antigenic determinant and does not cross-link unless multiple OSNs are anchored in the tumor cell membrane.

OSNs are not tightly bound or an integral membrane protein that penetrates through the membrane, otherwise the 1-phase butanol would not be expected to elute OSN molecules (45, 46). Moreover, the OSN molecule is not a proteolipid, since it was recovered in the aqueous phase in a water-organic solvent 2-phase system. OSN could be attached to membrane phospholipids by noncovalent interactions which may be disrupted by butanol. Alternatively, endogenous enzymes may be released by the extraction procedures and release covalently linked OSN.

The anchoring portion of the liberated OSN molecule may contain residual phosphatidyl groups, since butanol-solubilized molecules retain the ability to bind to phospholipid of vesicles or membranes (57, 58). Human OSNs share with immunoprotective tumor molecules of experimental tumors the unique physicochemical property of being selectively extracted with 2.5% butanol (32, 37–39, 41).

REFERENCES


Extraction of Human Organ-specific Cancer Neoantigens from Cancer Cells and Plasma Membranes with 1-Butanol

Nabil Labateya, David M. P. Thomson, Margaret Durko, et al.


Updated version
Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/47/4/1058

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.