Decreased 5' Nucleotidase Activity in Lymphocytes From Asymptomatic Sexually Active Homosexual Men and Patients With the Acquired Immune Deficiency Syndrome

By J.L. Murray, J.M. Reuben, C.G. Munn, P.W.A. Mansell, G.R. Newell, and E.M. Hersh

5' Nucleotidase (5'NT) is an ectoenzyme associated with the plasma membrane of most mammalian cells. Low 5'NT activity has been observed in peripheral blood lymphocytes from patients with immunodeficiency states. 5'NT activity was measured in null and T-enriched lymphocytes from asymptomatic homosexual men and from 20 men with various degrees of the acquired immune deficiency syndrome (AIDS). Asymptomatic homosexuals were self-referred because of their concern about AIDS and were not necessarily representative of homosexuals in the general population. Enzyme activity was significantly decreased in both null (7.0 ± 2.4 nmol/10^6 cells/h) and T-enriched (12.0 ± 6.0 nmol/10^6 cells/h) lymphocytes in homosexuals as compared to lymphocytes from age-matched heterosexual male and female controls (null = 10.8 ± 6.5 and T = 22.3 ± 10.6, P < .0001 and .008, respectively). Decreased activity was present regardless of whether the patients were asymptomatic, had prodromal symptoms such as fever, lymph node enlargement, weight loss and diarrhea, or had opportunistic infections or Kaposi's sarcoma. Homosexuals had a significantly higher fraction of lymphocytes expressing the activation antigens T10 (20% ± 3.3%) and la (13% ± 2.9%) than controls (11% ± 1.8% and 5% ± 0.8%, respectively, P < .05). They also had a significantly lower fraction of OKT4-positive helper lymphocytes than controls (22% ± 3.4% vs 35% ± 2.2%, P < .05). 5'NT activity in lymphocytes enriched for null cells from homosexuals correlated inversely with the percentage of la-positive lymphocytes (r = -.655; P < .02). There was no correlation between 5'NT activity and the percentage of T4- or T8-positive lymphocytes or the T4/T8 ratio. Moreover, 5'NT activity was significantly decreased in both OKT4 (P < .025) and OKT8 (P < .05) enriched lymphocytes in homosexuals compared to controls. The data suggest that decreases in 5'NT may be a generalized defect of the peripheral blood T lymphocytes from active homosexuals that is independent of increases or decreases in specific T subpopulations or clinical status. It may contribute to the pathogenesis of AIDS.

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THE ECTOENZYME, 5' nucleotidase (5'NT), catalyzes the dephosphorylation of nucleoside 5' monophosphates to yield the corresponding nucleoside. Lymphocytes are incapable of synthesizing purines de novo; hence, it has been postulated that 5'NT provides necessary nucleic acid metabolites that can be transported across the cell membrane. In thymus-derived (T) lymphocytes, 5'NT levels have been shown to correlate with the degree of cell maturity. A transient decrease in enzyme levels has been demonstrated in lymphocytes activated by antigens or mitogens. Decreased 5'NT activity has been demonstrated in the peripheral blood lymphocytes of patients with chronic lymphocytic leukemia, infectious mononucleosis, and persons with the congenital and common variable immunodeficiency syndromes. A deficiency in this enzyme may predispose to an immunodeficient state secondary to an arrest in lymphocyte differentiation and proliferation.

Since 1981, a syndrome of severe opportunistic infections, with or without Kaposi's sarcoma, and associated with immunodeficiency has been observed in young homosexual males. These patients are characterized as having defects in cellular immunity, including anergy to recall skin test antigens, lymphopenia, decreased lymphocyte blastogenic response to mitogens, low helper T cells, and an abnormal ratio of helper to suppressor T cells.

In this study, we have examined the level of 5'NT in both null and T-enriched peripheral blood lymphocytes from these and related individuals and have compared them to a control population. Lymphocyte surface markers were also examined using monoclonal antibodies. 5'NT was found to be low in all of the study subjects compared to normals. 5'NT activity did not correlate with the absolute percentage of helper cells, the helper:suppressor ratio, the percentage of T10-positive cells, the percentage of null-enriched cells, or the percentage of T-enriched cells. There was an inverse correlation, however, between 5'NT in null-enriched cells and the presence of the activation antigen la (HLA-Dr).

MATERIALS AND METHODS

Subjects

Twenty-nine homosexual males from 25 to 36 years of age were studied. Twelve heterosexual age-matched males and 15 age-
matched females served as controls. Nine of the homosexual males had been referred because of their concern about AIDS, but were clinically asymptomatic at the time of study. Six homosexuals had Kaposi's sarcoma, six had opportunistic infections, and eight had two or more prodromal symptoms (fever, night sweats, weight loss, fatigue, diarrhea, and lymphadenopathy). None of the patients had received chemotherapy or immunotherapy prior to study.

**Lymphocyte Separation**

Peripheral blood mononuclear cells (PBMCs) were separated from defibrinated whole blood by Ficoll-Hypaque density gradient centrifugation. PBMCs were depleted of monocytes and B cells and enriched for null and T cells using two comparable methods. In the first method, PBMCs were incubated for 45 minutes at 37 °C on nylon wool columns. Nonadherent lymphocytes were eluted with warm RPMI tissue culture medium containing 20% fetal calf serum (Grand Island Biologicals, Grand Island, NY), washed twice in RPMI, and counted. The nonadherent lymphocytes were separated into null and T-enriched fractions by incubation with neuraminidase (CalBioChem, La Jolla, Calif) treated sheep red blood cells (SRCBs) for ten minutes at 37 °C, followed by centrifugation and incubation for 60 minutes on ice. The pellet was resuspended, layered on Ficoll-Hypaque solution, and centrifuged for 15 minutes at 2,200 rpm. Cells at the interface (null-enriched) contained less than 7% ± 2% E rosetting cells, as determined by rerosetting. The SRBCs were lysed with 0.14 mol/L ammonium chloride, and the lymphocytes were resuspended in media. Greater than 93% ± 20% of these T-enriched cells formed E rosettes in both patient and control samples.

In the second method, PBMCs were depleted of monocytes and B cells by incubation for 60 minutes on polystyrene Petri dishes (Falcon Plastics, Oxnard, Calif) coated with affinity-purified goat anti-human Ig F(ab)2, (Cappel Laboratories, Cochranville, Pa). Nonadherent lymphocytes were then resuspended off the dish, washed twice in RPMI, and counted. Affinity-purified goat anti-mouse F(ab)2, (Tago, Burlingame, Calif) was coupled to ox erythrocytes (OEs) using chromic chloride. Nonadherent lymphocytes were incubated for 30 minutes at 4 °C with 5 μg anti-human Leu-1 monoclonal antibody (Becton Dickinson, Sunnyvale, Calif), washed, resuspended to 4 x 10⁶ cells/mL, and incubated with a 0.5% suspension of pretreated OEs for ten minutes. Lymphocytes were pelleted and incubated on ice for 60 minutes. Rosettes were gently resuspended, layered on Ficoll-Hypaque, and centrifuged at 1,400 rpm for 30 minutes. Interface cells were less than 12% ± 4% Leu-1-positive. Pelleted cells contained greater than 94% ± 30% OKT3-positive cells. OKT3 was used to examine for T cell purity in this case due to modulation of Leu-1 surface antigen expression in pelleted lymphocytes.

**Enrichment for OKT4- and OKT8-Positive Lymphocytes**

To obtain lymphocytes enriched for both OKT4 and OKT8, T cells were purified by using the neuraminidase-treated sheep red blood cell (E₆) technique described above. From 2 to 3 x 10⁶ E₆-purified T lymphocytes were then incubated with a 1:25 dilution of OKT8 (Ortho Diagnostics, Raritan, NJ) monoclonal antibody for 60 minutes on ice. After incubation, lymphocytes were resuspended to a concentration of 4 x 10⁶ cells/mL and separated into OKT4-enriched (> 83% ± 3%) and OKT8-enriched (> 92% ± 5%) lymphocytes, using the ox rosetting technique described above. Lymphocyte purity did not differ significantly between controls and homosexuals.

Ten microliters (0.5 μg) of the fluorescein-conjugated monoclonal antibodies OKT4, OKT8, (Ortho), anti-Leu-1 (Becton-Dickinson), and the unconjugated antibodies OKT9, OKT10, OK1a1 (Ortho), and anti-TAC (donated by Dr T.A. Waldmann) was added individually to each of seven tubes. Lymphocytes were incubated at 4 °C for 20 minutes and washed twice. In the case of unconjugated antibodies, indirect labeling with 50 μL of a 1:100 dilution of fluorescein-conjugated goat anti-mouse F(ab)'2, (Kallstadt Laboratories, Dallas) was performed. After another 20-minute incubation at 4 °C, cells were washed twice and the percentage of cells labeled with each antibody was determined using a Spectrum III flow cytometer (Ortho).

5' Nucleotidase Enzyme Analysis

5'NT levels in null and T-enriched lymphocytes were analyzed with a modification of the method of Van Larrhoven et al. Between 1,000 and 3,000 lymphocytes in 1 μL normal saline containing 0.5% bovine serum albumin (BSA) were added to individual wells of Terasaki microtiter plates (Falcon Plastics, Oxnard, Calif), frozen at −30 °C, and lypophilized. Five microliters of an enzyme reaction mixture containing 0.05 mol/L Tris/HCl (pH 8.5), 0.02 mol/L mgCl₂, 6.25 mmol/L 2-glycerophosphate (Sigma, St Louis), 0.6 mmol/L (8-β)³ adenosine monophosphate (AMP) (5P act 56 mCi; Amersham, New Haven, Conn), and 0.2% (vol/vol) Triton X100 was added to each of triplicate wells. Plates were incubated for two hours in a humidified atmosphere of 5% CO₂ in air, removed and placed in ice. One-microliter aliquots were aspirated and spotted on thin-layer chromatography (TLC) plates (Avicel-Analtech, Newark, Del). Plates were developed for three to four hours in the following solvents: EtOH (1 mol/L), NH₄, AC = 75:30 (vol/vol). Substrate and product were identified by UV fluorescence. The products were scraped off and added to individual 5-mL plastic scintillation vials (Fisher Scientific, Houston) along with 3 mL aquasol (Packard Instruments, Downers Grove, Ill). Radioactivity was measured in a scintillation counter (Packard), and the percentage of substrate that had converted to product was calculated. Enzyme activity was expressed as nmol/10⁶ lymphocytes/h.

**RESULTS**

**Percentage Yield and Number of Null and T-Enriched Lymphocytes in Homosexuals v Controls**

The average yield of nonadherent lymphocytes obtained from either the nylon column or panning techniques in both homosexuals and controls was 65% ± 6% of the total number of Ficoll-Hypaque density gradient-purified mononuclear cells obtained from 60 mL of whole blood. Less than 2% ± 1% of these cells were surface immunoglobulin (SIg) positive (B cells) or esterase positive (monocytes). The mean percent yield of null-enriched lymphocytes from either the neuraminidase-treated SRBCs or ox rosette method was higher in homosexuals (17% ± 3%) than in controls (9% ± 1.6%) (P < .002). Conversely, the mean percent yield of T-enriched cells was lower in homosexuals (22% ± 6%) than controls (45% ± 8%); (P < .006) (Table 1).

**5' Nucleotidase Activity**

5' Nucleotidase activity, as measured in both null and T-enriched subpopulations, is shown in Table 2 and Fig 1. Enzyme activity in nmol/10⁶ cells/h was higher in T-enriched fractions than in null-enriched
fractions in both homosexuals (P = .002) and controls (P < .0001). Homosexuals had statistically significant decreases in 5'NT levels in null (P < .01) and T (P < .005) subpopulations compared to controls. There was no difference in 5'NT levels between males and females in the control population. When patients were categorized according to clinical manifestations, mean 5'NT levels in T-enriched lymphocytes were significantly lower than controls in all patient subgroups, including asymptomatic individuals (Table 2 and Fig 1). 5'NT activity in null cells was also significantly lower than controls in all homosexual groups except those with opportunisitic infections.

**Lymphocyte Surface Marker Analysis**

Lymphocyte subpopulations were examined on non-adherent cells using monoclonal antibodies (Table 3). There was a significant decrease in OKT4-positive lymphocytes in homosexuals compared to controls (P < .05). OKT10- and OKIa1-positive lymphocytes were both increased in homosexuals compared to controls (P < .05). There were no significant correlations between null and T-enriched 5'NT activities, on the one hand, and helper-suppressor ratios on the other (Fig 2). When homosexuals were divided into those with a normal T4-T8 ratio (> 0.85) (N = 10) v those with an abnormal ratio (< 0.85) (N = 19), there was no difference in 5'NT activity in T-enriched cells (13 ± 6 v 12 ± 5) or null-enriched cells (7 ± 3 v 5 ± 2). Moreover, there was no correlation between 5'NT activity and the percent of OKT4, OKT8, OKT9, or OKT10 positive lymphocytes, null-enriched lymphocytes, or T-enriched lymphocytes (data not shown). However, as shown in Fig 3, a decrease in 5'NT in the null fraction correlated inversely with the percentage OKIa1-positive cells (r = -.655; P < .02).

**Table 1. Yield of Null- and T-Enriched Lymphocytes**

<table>
<thead>
<tr>
<th>Subject</th>
<th>N</th>
<th>Mean Percent Cell Yield (± SD)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homosexuals</td>
<td>29</td>
<td>17 ± 3</td>
<td>&lt; .002</td>
</tr>
<tr>
<td>Controls</td>
<td>27</td>
<td>9 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Null-enriched</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homosexuals</td>
<td>17</td>
<td>12 ± 2.4</td>
<td>&lt; .008</td>
</tr>
<tr>
<td>Controls</td>
<td>13</td>
<td>7 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>T-enriched</td>
<td>22</td>
<td>45 ± 8</td>
<td>&lt; .006</td>
</tr>
<tr>
<td>Homosexuals</td>
<td>15</td>
<td>40 ± 6</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>Controls</td>
<td>18</td>
<td>25 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. 5' Nucleotidase (5'NT) Activity**

<table>
<thead>
<tr>
<th>Subject</th>
<th>N</th>
<th>5'NT Activity* (± SD)</th>
<th>P Compared to Control</th>
<th>5'NT Activity (± SD)</th>
<th>P Compared to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27</td>
<td>10.8 ± 6.5†</td>
<td>—</td>
<td>22.3 ± 10.6</td>
<td>—</td>
</tr>
<tr>
<td>Homosexuals</td>
<td>29</td>
<td>7.0 ± 2.4</td>
<td>&lt; .008</td>
<td>12.0 ± 6.0</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>9</td>
<td>6.9 ± 3.3</td>
<td>&lt; .050</td>
<td>12.3 ± 5.9</td>
<td>&lt; .0050</td>
</tr>
<tr>
<td>Prodrome</td>
<td>8</td>
<td>7.9 ± 2.0</td>
<td>&lt; .050</td>
<td>15.0 ± 7.6</td>
<td>&lt; .0500</td>
</tr>
<tr>
<td>Opportunistic</td>
<td>6</td>
<td>8.7 ± 2.3</td>
<td>NS</td>
<td>9.8 ± 4.2</td>
<td>&lt; .005</td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
<td>6</td>
<td>6.0 ± 1.3</td>
<td>&lt; .005</td>
<td>10.0 ± 4.4</td>
<td>&lt; .0005</td>
</tr>
</tbody>
</table>

*Activity expressed as nmol/10⁸ cells/h.
†Mean activity ± 1 SD.

**5'NT Activity in OKT4- and OKT8-Enriched Lymphocytes**

In preliminary experiments, we measured 5'NT activity in lymphocytes enriched for both OKT4 and OKT8 (Table 4). Three controls and three homosexuals were studied. In the controls, 5'NT activity was significantly higher in the OKT8-positive subset than in the OKT4-positive subset (P < .005). Activity was significantly lower in both OKT4- and OKT8-positive subsets in homosexuals (OKT4 = 7.3 ± 0.6; OKT8 = 24.4 ± 17) compared to controls (OKT4 = 10.8 ± 1.7; P < .025; OKT8 = 63.1 ± 18.3; P < .05).

**DISCUSSION**

The most interesting finding of this study was that homosexual males had significant decreases in both null and T lymphocyte 5'NT activity compared to normal heterosexual controls, regardless of clinical status. In addition, there was no significant correlation between low 5'NT levels and either the percentage of...
Table 3. Lymphocyte Subpopulations in Patient and Control T-Enriched Fractions

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Leu-1</th>
<th>T4</th>
<th>T8</th>
<th>T9</th>
<th>T10</th>
<th>Ia</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>60 ± 3.7</td>
<td>35 ± 2.2</td>
<td>31 ± 2.0</td>
<td>2 ± 0.6</td>
<td>11 ± 1.8</td>
<td>5 ± 0.8</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>Patient</td>
<td>20</td>
<td>50 ± 7.0</td>
<td>22 ± 3.4*</td>
<td>39 ± 4.3</td>
<td>2 ± 0.7</td>
<td>20 ± 3.3*</td>
<td>13 ± 0.9*</td>
<td>3 ± 0.9</td>
</tr>
</tbody>
</table>

*Patient vs normals: P < .05.

Table 4. 5'NT Activity in OKT4- and OKT8-Enriched Lymphocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>OKT4</th>
<th>OKT8</th>
<th>H/S Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12.0 ± 3.3*</td>
<td>76.3 ± 25.5</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>8.9 ± 1.6</td>
<td>42.2 ± 1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>11.5 ± 0.7</td>
<td>70.8 ± 8.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Mean</td>
<td>10.8 ± 1.7</td>
<td>63.1 ± 18.3</td>
<td></td>
</tr>
</tbody>
</table>

| Homosexuals |       |       |           |
| 1      | 8.0 ± 1.6   | 38.2 ± 10.3 | 0.63      |
| 2      | 7.0 ± 0.5   | 5.0 ± 0.8    | 0.61      |
| 3      | 7.0 ± 0.7   | 30.1 ± 1.2   | 0.83      |
| Mean   | 7.3 ± 0.6   | 24.4 ± 17.0  |           |

P value† < .025 < .050

*Mean ± SD of triplicate values.
†Homosexuals vs controls.

Fig 2. Correlation between the helper-suppressor ratio (T4:T8) in homosexual males and 5'nucleotidase activity. No significant correlation was observed (r = -.100; P > .05).

OKT4-positive cells or the ratio of OKT4 + to OKT8 + cells. Enzyme activity was significantly decreased in both OKT4- and OKT8-enriched subsets from homosexuals relative to controls. This suggested that the decrease in enzyme activity was related to an intrinsic T cell and T cell precursor defect, rather than to increase or decrease of a specific helper or suppressor subpopulation.20

Although levels of ecto-5'NT activity have been reported higher in intact B cells than in T cells,21 other studies suggest that differences in enzyme activity are more likely related to the stage of lymphocyte maturation rather than to their B or T cell character.22,23 The percentage of Slg+ B cells in the control null and T-enriched fractions (null = 2.1% ± 0.5%; T = 1.1% ± 0.8%) was similar to that found in homosexuals (null = 1.6% ± 1.3%; T = 0.8% ± 0.5%). Hence, it is unlikely that the differences observed between heterosexuals and homosexuals in 5'NT activity were due to differences in B cell number. The enzyme activity reported
here was higher than that observed in null cells in a previous report. However, this most likely relates to the fact that whole cell 5'NT activity was measured in our assay rather than the production of ecto-5'NT by intact cells.

The methods for measuring ecto-5'NT activity have been compared, and it was shown that 5' [32P]AMP is the most suitable radiolabeled substrate for measuring ecto-5'NT activity in intact cells. The use of other radiolabeled substrates results in falsely low levels of ecto-5'NT due to rapid phosphorylation of adenosine. This may account for discrepancies among previous studies in the literature reporting widely differing 5'NT activities for different cell types. Our measurements of 5'NT activity in a nonviable cell preparation represent a combination of cytosolic and ecto-5'NT present.

Decreases in 5'NT activity could be related to abnormal or defective lymphocyte differentiation or to different degrees of activation in patients vs normals. In this study, as in others, homosexuals had a greater percentage of OKT10- and OKIa-positive lymphocytes. Since significant increases in lymphocytes expressing the activation antigens T9 (transferrin receptor) or TAC (IL-2) receptor were not observed, it was concluded that decreases in 5'NT activity were related to abnormal or defective lymphocyte maturation. Recent studies in AIDS have demonstrated evidence of thymic destruction. This finding, coupled with the existence of immature peripheral blood T cells, lends further support to this hypothesis. The so-called null cell fraction contains both B and T cell precursors, which could explain the lower 5'NT activity observed in null lymphocytes compared to T lymphocytes in both controls and homosexuals, as well as the positive correlation between increased 1a expression and decreased 5'NT levels (Fig 2). However, it is also possible that the increase in T10 and 1a expression along with the decreased 5'NT activity could relate to lymphocyte membrane perturbation, as has been described by Mittler et al.

The clinical relevance of decreases in lymphocyte 5'NT activity in active homosexuals is unknown. Low enzyme activity is most likely a result, rather than a cause, of immunodeficiency in this group. The enzyme is one of the concanavalin A receptors, and therefore, may be related to the abnormal proliferation of lymphocytes to Con-A in AIDS. Decreased ecto-5'NT activity has also been reported in lymphocytes from patients with immunodeficiency diseases and lymphoproliferative disorders. Thompson et al noted that patients with hypogammaglobulinemia have decreased percentages of OKT4-positive lymphocytes and increased percentages of OKT8-positive lymphocytes, similar to what has been observed in homosexuals. Ecto-5'NT activity was decreased in both OKT4 and OKT8 subpopulations, as noted in this study. In addition, patients with hypogammaglobulinemia had lymphocytes that were histochemically negative for 5'NT and were capable of suppressing mitogen-induced IgG synthesis by normal B cells, similar to what has been reported in AIDS. A generalized decrease in 5'NT activity has also been described in chronic lymphocytic leukemia. Furthermore, this decrease in activity was present in both T cells and malignant B cells from the patients. 5' Nucleotidase was also shown to be decreased in individual lymphocytes during the active phase of infectious mononucleosis. This finding could have clinical relevance, because 24 of the 29 homosexuals studied had positive titers for Epstein-Barr virus of greater than 1:160, including seven of the nine who were asymptomatic.

Since 5'NT was also significantly decreased in asymptomatic homosexuals, the assay may have clinical utility as a screening test for high-risk populations, similar to serum thymosin α-1 levels. Nevertheless, considerable caution should be exercised in interpreting these findings, because it is unlikely that the low enzyme activity observed in our asymptomatic homosexual group is representative of values that would be obtained in randomly screened homosexuals from the general population. Interestingly, seven of the nine sexually active, asymptomatic homosexuals had helper-suppressor cell ratios greater than one, which is actually higher than that previously reported in the literature or in 25 asymptomatic individuals recently screened at this institution who had been exposed to persons with AIDS (mean H-S ratio, 0.63).

Low lymphocyte 5'NT activity may be a selective defect, since it has been previously demonstrated that lymphocytes from cancer patients and a wide variety of infections have normal enzyme levels. In order to validate whether these findings are specific for active homosexuals, in general, however, other control groups, including patients with symptomatic viral infections, must be studied. Additional long-term prospective studies of lymphocyte 5'NT levels in randomly screened asymptomatic homosexuals, along with measurements of enzyme activity in other closely related clinical syndromes, are needed before questions regarding specificity can be sufficiently answered.

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