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Naturally Developing Memory T Cell Xenoreactivity to Swine Antigens in Human Peripheral Blood Lymphocytes

Carsten V. Hartig,* Gary W. Haller, † David H. Sachs, † Shannon Kuhlenschmidt,* and Peter S. Heeger2*

Naturally developing xenospecific Abs are well-documented barriers to xenograft transplantation in humans, but whether analogous xenoreactive T cell immunity develops is not known. We used an enzyme-linked immunospot assay to determine the frequency and cytokine profiles of xenoreactive PBLs from a panel of human volunteers. Because naïve T cells produce only IL-2 in short term culture, IFN-γ production by this approach is a measure of a memory immune response. Stimulation of human PBLs or purified T lymphocytes with stimulator cells from inbred swine revealed a high frequency of IFN-γ producers with 5-fold fewer IL-2 producers. In contrast, lymphocytes obtained from neonatal umbilical cord blood contained swine-specific IL-2 producers but few IFN-γ producers, which is what one would expect to find with a naïve phenotype. Moreover, PBLs from adults with a history of abstention from pork consumption responded to swine cells with a significantly lower frequency of IFN-γ producers than PBLs from adults with unrestricted diets did, suggesting that pork consumption may result in priming of swine-specific T cell immunity. Our findings provide the first evidence for naturally occurring xenospecific T cell immunity in humans. The detected strength of this memory response suggests that it will present a formidable barrier to transplantation of swine organs. The Journal of Immunology, 2000, 164: 2790–2796.

The limited supply of available human organs has resulted in an increasing clinical interest in the use of xenografts for transplantation, and organs obtained from domestic swine are considered potentially useful in this regard (reviewed in Ref. 1). Because immune-mediated rejection is a major factor limiting the widespread implementation of xenogeneic transplantation (1, 2), a thorough understanding of the xenoreactive, human anti-swine immune response is essential. Recently reported studies on the humoral and cellular mediators of human anti-swine xenoreactivity have provided new insights into the mechanisms of xenograft rejection. It is now well established, for example, that humans (and Old World primates) naturally develop high titers of xenospecific Abs directed toward swine endothelial Ags (3, 4). These Abs recognize one dominant gal (α1,3) gal determinant and induce complement-mediated hyperacute rejection of transplanted organs (5). A significant amount of experimental effort by a number of laboratories has been directed at overcoming and/or bypassing this natural humoral immunity, and several promising approaches are under study (1, 6–11).

In addition to the human anti-swine humoral immune response, T lymphocytes are capable of mediating a slower (but effective) form of xenograft rejection and are likely to be potent barriers to effective xenotransplantation once the problems of xenobody-mediated rejection are overcome (12–15). In an analogous fashion to naturally developing humoral immunity, it is possible that humans develop memory T cell immunity toward swine Ags. Such a finding might have significant implications for survival of transplanted xenografts. Consistent with the hypothesis that primed T cell immunity can adversely affect graft survival, we have recently used a highly sensitive enzyme-linked immunospot (ELISPOT) assay to show that human recipients of kidney allografts with a high pretransplant frequency of donor-specific memory cells are at high risk of posttransplant rejection episodes (16).

We have now applied this same approach to peripheral blood T cells of human adults to determine whether primed memory cells specific for swine Ags exist in the absence of known sensitization. The results of these studies are in this report and provide the first evidence for naturally developing T cell immunity to swine Ags in humans.

Materials and Methods

Isolation and purification of human PBLs and T cells

Peripheral blood samples were obtained from 14 adult volunteers with unrestricted diets and from 42 patients from families being evaluated for renal transplantation at the University Hospitals of Cleveland. In addition, we obtained blood samples from 14 adults (four orthodox Jews, eight Muslims, and two Hindu individuals) who, for religious reasons, had a history of abstaining from pork consumption. Blood samples were also obtained from four infants 6–24 mo of age, and neonatal cord blood samples from 17 patients were obtained after normal vaginal deliveries. Mononuclear cells were isolated from 5–20 ml of blood by standard Isopaque (Robbins Scientific, Sunnyvale, CA) centrifugation (16). Viable cells were counted using an immunofluorescence microscope in the presence of acridine orange/ethidium bromide. In some experiments, human T cells were enriched using commercially available T cell isolation columns.

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(R&D Systems, Minneapolis MN). All studies were performed under the approved guidelines set forth by the internal review board for human studies at University Hospitals of Cleveland and the Cleveland Veterans Affairs Medical Center.

**Mouse studies**

C57BL/6 mice (H-2b) were purchased from The Jackson Laboratory, Bar Harbor ME. A total of 5–10 × 10⁶ swine PBLs were injected i.p. into mouse recipients. Fourteen days later, splenic T cells were isolated using murine T cell isolation columns (R&D Systems) and tested in murine ELISPOT recall assays (17).

**Determination of MHC I and MHC II phenotypes**

HLA phenotypes were determined by standard clinical typing techniques (16). Ags encoded by HLA class I loci (A, B) were identified by the basic microlymphocytotoxicity assay, using local antisera as previously described. Class II alleles were determined by sequence-specific priming and PCR using a Purigen DNA isolation kit (Genta Systems, Minneapolis, MN) and sets of primers (One Lambda, Canoga Park, CA) specific for one or a few alleles, as previously described (16).

**Preparation of stimulator cells**

PBLs obtained from inbred SLA^aa^, SLA^cc^, and SLA^dd^ strains of miniature swine were isolated from blood samples by Isoprep centrifugation. In some experiments stimulator swine cells or human cells were prepared by treatment with 50 μg/ml mitomycin C (Boehringer Mannheim, Indianapolis, IN) for 30 min before three washes in HBSS (16).

**ELISPOT assay**

Ninety-six-well ELISPOT plates (Cellular Technology, Cleveland, OH) were coated with capture Abs for IL-5 (TRFK5; isolated from hybridoma in our laboratory; 5 μg/ml), IL-4 (8D4-8; PharMingen, San Diego, CA; 2 μg/ml), IL-2 (5334.21; R&D Systems; 6 μg/ml), or IFN-γ (2G1; Endogen, Woburn, MA; 4 μg/ml) in PBS overnight at 4°C. The plates were then blocked with PBS plus 1% BSA and washed with PBS. A total of 300,000 responder PBLs were added to each well in 100 μl complete RPMI medium (16). FCS was obtained from HyClone (Logan, UT). The PBLs or purified T cells were added to the ELISPOT wells and activated in vitro with swine stimulator lymphocytes, sonicates of swine stimulator lymphocytes (prepared by sonicating 2 × 10⁶ swine cells in 1 ml RPMI with 10 1-s pulses using a Fisher Scientific cell sonicator (Pittsburgh, PA) before freeze-thaw), syngeneic or allogeneic human PBLs, or PHA (10 μg/ml; bottom row). Swine stimulators ± PHA did not produce detectable cytokine (data not shown).

Human cells. Representative ELISPOT wells shown in Fig. 1 revealed essentially no detectable responses from unstimulated human PBLs. In contrast, distinct spots representing cytokine produced by individual cells were noted when the PBLs were mixed with swine stimulators. As can be seen, IFN-γ- and IL-2-producing cells dominated the response, although some IL-5 and IL-4 producers were also detected. Mitogen stimulation with PHA served as a positive control and induced production of all cytokines tested.

The frequencies and cytokine profile of swine-specific PBLs from a panel of 14 adult human volunteers (with unrestricted diets) are depicted in Fig. 2, A–C. The cytokine profile of the swine-

**Results**

We initially characterized the frequency and cytokine profiles of swine-reactive PBLs from a panel of adults with unrestricted diets using an ELISPOT assay. Using this approach, cytokine production was detected within 24–48 h of stimulation in vitro, a time period too short for T cell proliferation and differentiation. We have previously demonstrated that this assay has single-cell resolution and that detection of IFN-γ, IL-4, and IL-5 production over this interval represents cytokine produced by primed cells (16). Human PBLs were tested for cytokine production in response to stimulator PBLs obtained from inbred SLA^aa^, SLA^cc^, and SLA^dd^ miniature swine. Preliminary studies demonstrated that the Abs used for the human ELISPOT did not crossreact with swine cytokines (data not shown), so all detected responses derived from the

**Cytokine**

- **A**: SLA^aa^ (IFN-γ, IL-2, IL-4, IL-5).
- **B**: SLA^cc^ (IFN-γ, IL-2, IL-4, IL-5).
- **C**: SLA^dd^ (IFN-γ, IL-2, IL-4, IL-5).
- **D**: Allo (IFN-γ, IL-2, IL-4, IL-5).

**FIGURE 1.** ELISPOT detection of human anti-swine immunity. Representative IFN-γ, IL-2, IL-4, and IL-5 ELISPOT wells using 3 × 10⁶ human responder PBLs per well plus medium alone (top row), 3 × 10⁶ swine stimulator cells (middle row), or PHA (10 μg/ml; bottom row). Swine stimulators ± PHA did not produce detectable cytokine (data not shown).

**FIGURE 2.** Frequency and cytokine profiles of swine-specific PBLs and alloreactive PBLs from adults. PBLs from 14 adult volunteers with unrestricted diets were tested in cytokine ELISPOT assays for reactivity to stimulator cells from inbred SLA^aa^ (A), SLA^cc^ (B), and SLA^dd^ (C) miniature swine. D: Results of cytokine ELISPOT assays for 42 different individuals tested against allogeneic stimulator cells that were mismatched at five or six A, B, and DR loci. Each point represents the mean value of duplicate wells for a single individual (<10% variability among wells). The number adjacent to each set of data points is the mean frequency per million cells for all individuals tested. PHA stimulation of responder cells induced >200 spots/well for each cytokine (data not shown).
specific PBLs was overwhelmingly dominated by IFN-γ and IL-2 producers for all three haplotypes tested (SLA\textsuperscript{aa}, SLA\textsuperscript{ab}, and SLA\textsuperscript{bb}). The frequency of swine-specific IFN-γ-producing cells was 10–850/million cells (a frequency of 1/100,000 to 1/1,200) with a mean value ranging from 264 to 313/million, which is consistent with a potent memory response to swine Ags in >90% of the adults tested. PBLs from <10% (1 of 14) of the samples tested did not produce swine-specific IFN-γ ELISPOTs, although positive control PHA stimulation demonstrated that these cells were capable of producing IFN-γ.

Results of analogous studies of alloreactivity, using PBLs from a panel of adults undergoing evaluation for renal transplantation in response to allogeneic stimulators mismatched at five or six of the loci at A, B, and DR, are shown in Fig. 2D for comparison (all of these patients had “panel of reactive Ab” values of <5%). Like the xenoresponse and consistent with our previously published studies (16), allodextral PBLs predominantly produced IFN-γ and IL-2 but additionally produced some IL-5. The mean detected frequency for allodextral IFN-γ producers was 157/million (range, <5–850), which was lower than the detected frequencies for xenoreactive cells, but there was significant overlap between the groups. Overall, the data suggest that naturally developing cellular immunity to swine Ags is present in adult humans and that the frequency of xenoreactive PBLs is ~2-fold higher than the frequency of allodextral PBLs.

In vitro priming of human PBLs induced a marked increase in the frequency of xenoreactive cytokine producers in a recall assay (Table I and Fig. 3) up to 30-fold over the baseline response. Although this primed response remained dominated by IFN-γ-producing cells, significant numbers of IL-4 and IL-5 producers were detectable as well. Priming with allostimulator cells from an unrelated, fully HLA-mismatched individual also resulted in a significant increase in the number of cytokine producers over baseline, but the frequency of primed alloreactive cells remained invariably lower than in the xenoprimered response (Table I). Consistent with previously published studies using swine-specific CD4\textsuperscript{+} T cell lines (13), priming of PBLs with SLA\textsuperscript{aa} or SLA\textsuperscript{ab} stimulators resulted in vigorous recall responses that were generally crossreactive to each other (Table II). Priming with SLA\textsuperscript{ab} induced recall reactivity to both SLA\textsuperscript{ab} and SLA\textsuperscript{aa}, with little crossreactivity to SLA\textsuperscript{cc}. Priming with SLA\textsuperscript{aa} induced a crossreactive response to both SLA\textsuperscript{aa} and SLA\textsuperscript{cc}, although the recall response to SLA\textsuperscript{aa} was significantly stronger (~2-fold) than that to SLA\textsuperscript{cc} (p < 0.05). In contrast, priming with SLA\textsuperscript{cc} stimulators resulted in SLA\textsuperscript{cc}-specific immunity with relatively low crossreactivity to SLA\textsuperscript{aa} or SLA\textsuperscript{ab} (Table II). Moreover, in vitro priming with SLA\textsuperscript{aa} stimulators resulted in xenospecific immunity with 10- to 30-fold fewer cells reactive to control allogeneic stimulator cells (Fig. 3), further demonstrating the specificity of the induced responses.

To confirm that the detected cytokines were produced by xenoreactive T lymphocytes and to determine whether the response represented direct vs indirect recognition of swine Ags, we next isolated purified T cells from the PBLs. The purified T cells were

### Table I. Comparison of allo- and xenoreactivity in human PBLs before and after in vitro priming\textsuperscript{a}

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>PBLs</th>
<th>Stimulator</th>
<th>Recall ELISPOTs (spots/10\textsuperscript{6} cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IFN-γ</td>
</tr>
<tr>
<td>1 Fresh</td>
<td>SLA\textsuperscript{ab}</td>
<td>232</td>
<td>85</td>
</tr>
<tr>
<td>1 Primed</td>
<td>SLA\textsuperscript{ab}</td>
<td>1793</td>
<td>628</td>
</tr>
<tr>
<td>1 Fresh</td>
<td>Allo (1 vs 2)</td>
<td>83</td>
<td>13</td>
</tr>
<tr>
<td>1 Primed</td>
<td>Allo (1 vs 2)</td>
<td>815</td>
<td>108</td>
</tr>
<tr>
<td>2 Fresh</td>
<td>SLA\textsuperscript{ab}</td>
<td>165</td>
<td>52</td>
</tr>
<tr>
<td>2 Primed</td>
<td>SLA\textsuperscript{ab}</td>
<td>1245</td>
<td>23</td>
</tr>
<tr>
<td>2 Fresh</td>
<td>Allo (2 vs 1)</td>
<td>98</td>
<td>32</td>
</tr>
<tr>
<td>2 Primed</td>
<td>Allo (2 vs 1)</td>
<td>2390</td>
<td>18</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Freshly isolated PBLs from two adult volunteers were either directly tested in cytokine ELISPOT assays or were primed in vitro with mitomycin C-treated stimulator cells for 6 days and then tested in specific recall cytokine ELISPOT assays. Stimulator cells were either xenogeneic PBLs from inbred swine (SLA\textsuperscript{ab}) or fully HLA-mismatched human PBLs (Allo). The HLA alleles expressed by individual 1 are A2, A28, B13, B14, DR1, DR7, and the HLA alleles expressed by individual 2 are A1, A23, B8, B44, DR17, 52. Spot frequencies represent the mean values of duplicate wells counted by computer-assisted image analysis. There was ~10% variation among wells. The findings are representative of three individual experiments.

![FIGURE 3. Frequency and specificity of in vitro primed human anti-swine cellular immunity.](http://www.jimmunol.org/Downloadedfrom)
tested in response to swine stimulators as a measure of direct xenoreactivity. To detect indirect xenoreactivity, the T cells were stimulated with mitomycin C-treated syngeneic PBLs (as a source of APCs) plus a swine cell sonicate, a process analogous to our previously published studies of murine indirect alloreactivity (17).

As shown in Fig. 4, the frequency of detected cytokine producers using T cells (>92% CD3<sup>+</sup> by flow cytometry; data not shown) was significantly greater than that detected using unfractionated PBLs, (35–40% CD3<sup>+</sup> by flow cytometry; data not shown), which is consistent with enrichment of a cytokine-producing T cell population. The T cells did not respond to syngeneic stimulators ± swine sonicates (and did not respond to sonicates alone), suggesting that indirect reactivity does not contribute significantly to this naturally developing anti-swine cellular immune response.

As a positive control to confirm that the swine-derived sonicates can elicit a recall immune response if one is present, we isolated purified murine splenic T cells 2 wk after an i.p. injection of swine cells and tested them in ELISPOT assays. As shown in Fig. 4C, the purified T cells did not respond to syngeneic APCs or to swine sonicates alone but did respond to syngeneic APCs plus swine sonicates, a response which is consistent with indirect xenoreactivity. Thus, our ability to detect human T cell production of IFN-γ in response to intact swine cells but not in response to syngeneic APCs plus swine sonicates (Fig. 4, A and B) is consistent with direct recognition of swine cells.

A comparison of frequencies and full cytokine profiles for freshly isolated T cells responding through the direct pathway to xenoantigens and alloantigens for three representative individuals is shown in Table III. As can be seen, direct xenoreactivity was detectable at a higher frequency than direct alloreactivity was, although both types of stimulators induced similar IFN-γ-dominated cytokine profiles. In sum, our data demonstrate that the detected, naturally developing memory xenoresponse derived from T cells was overwhelmingly dominated by direct recognition of xenotagonists and was detectable at a higher frequency than the response to alloantigens was.

If the swine-specific T cell immune response in adults is a result of environmental exposure to swine Ags and/or crossreactive memory, then PBLs from neonates without previous environmental exposure should not contain significant frequencies of swine-specific IFN-γ-producing PBLs. Consistent with this hypothesis, neonatal cord blood lymphocytes contained similar frequencies of IL-2 producers, but contained 10- to 50-fold fewer IFN-γ producers than adult PBLs did when stimulated with swine cells (Fig. 5A), which is consistent with a naive cytokine-producing phenotype. Importantly, in vitro priming of neonatal cord blood lymphocytes with swine stimulator cells induced strong IFN-γ production in a recall response (Fig. 5B), demonstrating that the neonatal cells were capable of developing into memory, swine-reactive IFN-γ producers. Restimulation with allogeneic PBLs (after in vitro priming with xenostimulators) yielded a 10- to 15-fold lower frequency of IFN-γ producers (75–180/10<sup>6</sup>; data not shown) compared with the swine-specific responses, confirming the specificity of the induced response. We further tested PBLs obtained from several infants (ages 6–24 mo) and similarly noted that the anti-swine immune response was of low frequency and was dominated by IL-2, which is consistent with the response of a naive phenotype (mean values: 13/10<sup>6</sup> cells for IFN-γ, 51/10<sup>6</sup> cells for IL-2, 13/10<sup>6</sup> cells for IL-4, and 6/10<sup>6</sup> cells for IL-5; n = 4; data not shown). In sum, the data suggest that the naturally developing, anti-swine cellular immunity detected in adults is a result of environmental exposure.

Although crossreactive anti-swine immunity could develop after exposure to a variety of environmental Ags (e.g., viruses, transfusions, or pregnancy) as is hypothesized for alloimmunity (18, 19), one potential source of exposure to swine Ags is pork consumption. If consumption of pork is an important factor in developing anti-swine cellular immunity, then individuals without pork consumption might be expected to have naive responses analogous to

### Table II. Specificity of xenoreactive PBLs after in vitro priming<sup>a</sup>

<table>
<thead>
<tr>
<th>Priming Stimulator Cells</th>
<th>SLA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SLA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SLA&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprimed</td>
<td>73</td>
<td>31</td>
<td>140</td>
</tr>
<tr>
<td>SLA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4223</td>
<td>1067</td>
<td>1863</td>
</tr>
<tr>
<td>SLA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>770</td>
<td>1333</td>
<td>400</td>
</tr>
<tr>
<td>SLA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>717</td>
<td>210</td>
<td>997</td>
</tr>
</tbody>
</table>

<sup>a</sup> Freshly isolated human PBLs from a healthy adult were tested directly in IFN-γ ELISPOT assays against swine stimulators (unprimed) or were stimulated for 5 days in vitro with either SLA<sup>a</sup>, SLA<sup>c</sup>, or SLA<sup>d</sup> swine stimulator cells before a recall IFN-γ ELISPOT to all three stimulators. Spot frequencies represent the mean values of duplicate wells counted by computer-assisted image analysis. There was ~10% variation among wells. The findings are representative of three individual experiments performed using two different responders.
those found in neonates and infants. To provide some insight into this question, we tested for swine-specific immune responses using PBLs obtained from 14 adults with a dietary history devoid of pork consumption (Fig. 6). Notably, the PBLs from these individuals produced significantly lower frequencies of IFN-γ than those from the adults with unrestricted diets (p < 0.02; Fig. 6), although the detected frequencies of IL-2, IL-4, and IL-5 producers were not significantly different between the groups. Six of the fourteen individuals (43% of those tested) in fact produced IL-2 with essentially no IFN-γ (<20 spots/10^6 cells), which is consistent with a naive cytokine phenotype (Fig. 6A) and is similar to the responses detected in the neonatal samples (Fig. 5). Importantly, however, the PBLs from these individuals could be primed in vitro to produce IFN-γ in response to swine cells, confirming their ability to develop into a SLA-specific memory phenotype (Fig. 6B).

**Discussion**

It is now well established that naturally developing human anti-swine xenoantibodies are significant barriers to effective xenotransplantation (3, 4). This humoral immunity is thought to participate in hyperacute and delayed acute xenograft rejection through activation of complement with subsequent vascular thrombosis and ischemia (1, 5). A large amount of effort expended by a number of research laboratories and biotechnology companies has resulted in several novel strategies aimed at bypassing these effects, including production of genetically altered xenografts resistant to xenoantibody/complement-mediated injury (1, 6–11). Once these humoral barriers to xenotransplantation have been effectively controlled, the effects of xenoreactive T lymphocytes as mediators of xenograft rejection will need to be fully addressed.

Recent studies have shown that T lymphocytes recognize xenantigens through both the direct and indirect pathways of xenorecognition and that T cells can mediate xenograft rejection in the absence of Abs (12–15, 20). Such findings clearly show that effective control of T cell immunity will be required before xenotransplantation can become a routinely efficacious modality for therapy of end-stage organ failure.

Our data provide strong evidence for the presence of naturally developing memory T cell immunity directed toward swine Ags in the peripheral blood of human adults. Previously, we have shown that the ability to detect IFN-γ production by ELISPOT in short term culture is a measure of Ag-specific memory, and furthermore, it is significantly more sensitive than other available assays for detection of Ag-specific immunity in humans (16). Using this ELISPOT approach, we now demonstrate that swine-specific, IFN-γ-producing T cells are readily detectable in adult human subjects (Figs. 1 and 2) and that the responses tend to be more frequent than alloresponses determined by the same techniques and in the same individuals (Fig. 2 and Table I). Moreover, the detectable responses were shown to reflect direct recognition of xenantigens in that purified T cells produced the IFN-γ when directly stimulated with swine stimulator cells (Fig. 4). In contrast to blood from adults, neonatal cord blood and blood samples from infants contained low frequencies of IL-2-dominated, swine-specific PBLs, which is consistent with a naive cytokine phenotype (Fig. 5). These cells produced IFN-γ in 24-h recall ELISPOT assays when

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Table III. Frequency and cytokine profiles of freshly isolated human T cells responding to xenogeneic and allogeneic stimulators (spots/10^6 cells)

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>SLA^aa</th>
<th>Allo</th>
<th>SLA^aa</th>
<th>Allo</th>
<th>SLA^aa</th>
<th>Allo</th>
<th>SLA^aa</th>
<th>Allo</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1080</td>
<td>40</td>
<td>288</td>
<td>124</td>
<td>8</td>
<td>12</td>
<td>140</td>
<td>60</td>
</tr>
<tr>
<td>B</td>
<td>256</td>
<td>120</td>
<td>72</td>
<td>12</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>212</td>
<td>120</td>
<td>24</td>
<td>55</td>
<td>8</td>
<td>17</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*Enriched human T cells were isolated (>92% CD3^+ by flow cytometry; data not shown) from human adult PBLs using commercially available T cell isolation columns (R&D Systems) and were tested in cytokine ELISPOT assays in response to xenogeneic (SLA^aa) or allogeneic stimulator cells (mismatched at four to six alleles at the A, B, and DR loci). Spot frequencies represent the mean values of duplicate wells counted by computer-assisted image analysis. There was ~10% variation among wells. The findings are representative of three individual experiments.

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**FIGURE 5.** Frequency and cytokine profiles of anti-swine xenoreactive PBLs from neonatal cord blood. A. Lymphocytes isolated from 14 individual neonatal cord blood samples were tested in cytokine ELISPOT assays in response to SLA^aa stimulator cells. Each point represents the mean value of duplicate wells for a single individual (<10% variability among wells). The number adjacent to each set of data points is the mean frequency per million cells for all individuals tested. Similar results were noted in response to SLA^ads or SLA^add stimulator cells (data not shown). PHA stimulation of responder cells induced >50 spots/well for IFN-γ and >200 spots/well for IL-2, IL-4, and IL-5 (data not shown). B. Five of the freshly isolated neonatal cord blood samples were immediately tested in IFN-γ ELISPOT assays in response to SLA^aa stimulators (Fresh) or were primed in vitro with swine SLA^aa stimulators for 6 days and then retested in a recall assay against SLA^aa stimulator cells. Each point represents the mean value of duplicate wells for a single individual (<10% variability among wells). Specificity controls revealed 75–180 spots/million when the SLA^aa-primed cells were tested in response to allogeneic stimulators (data not shown).
PHA stimulation of responder cells induced significant difference between groups for the IL-2, IL-4, or IL-5 responses. Dashed lines connect the results of specific and control recall responses for a given individual. The xenospecific cytokine profile in our studies was overwhelmingly dominated by IFN-γ, with few IL-4 and IL-5 producers. Furthermore, we did not detect xenospecific T cell production of IL-10 (data not shown). Several experimental models of xenotransplantation have implicated the type 2 cytokines IL-4, IL-5, and IL-10 as important mediators of the rejection process (1, 25). Our data do not dispute these findings but instead suggest that the pretransplant cytokine profile is dominated by IFN-γ.

The reason for the detected high frequency of swine-specific memory cells remains to be further established, although our findings provide potential insight into this issue. First, the finding that lymphocytes from neonates and infants do not have significant memory responses to swine Ags (Fig. 4) suggests that this immune response develops through environmental exposure. T lymphocytes that were primed to a variety of environmental Ags (i.e., through blood transfusion, immunization, pregnancy, viral infection, etc.) could crossreact with swine Ags. In addition, our data suggest that, at least for some individuals, pork consumption may contribute to the development of the memory T cell response. We found that the PBLs from individuals with a history of abstention from pork consumption had fewer swine-specific, IFN-γ-producing cells than the PBLs from adults with unrestricted diets (Fig. 6). Moreover, PBLs from several of these individuals expressed a naive cytokine phenotype (that had a low frequency and was IL-2 dominated), which was similar to the neonatal response (Fig. 6). However, once again the PBLs from those with a history of abstention from pork consumption could be primed in vitro to specifically produce IFN-γ, confirming that the cells were capable of producing this cytokine under some stimulatory conditions. The detection of IFN-γ-producing, swine-specific PBLs in some of the subjects who abstained from eating pork may be attributable to a variety of factors including unknown pork consumption or cross-reactive immunity after exposure to other environmental Ags.

Our data further demonstrate that the anti-swine, cytokine-producing cellular immune response in adults with unrestricted diets is somewhat more frequent (~2-fold) than alloimmune response and can be detected in frequencies as high as 1/1200 PBLs (Figs. 2 and 3 and Table III). These results are generally consistent with those published previously by Murray et al. (24) for IL-2-producing human T cells, although our detected frequencies tended to be slightly lower than those previously reported. These modest differences are likely related to technical issues such as the use of 96-h limiting dilution analysis, which may permit in vitro priming/clonal expansion (24), instead of our 24-h ELISPOT approach.

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Similarly, although indirect xenoreactivity has been demonstrated to be a prominent component of the immune response during xenograft rejection in some animal models (1, 13, 14), our experiments suggest that the xenoresponse in humans is dominated by direct xenoreactivity. Purified T cells responded to swine stimulators but did not respond to a sonicated preparation of swine cells with or without the addition of syngeneic PBLs (Fig. 3, a
method previously shown by our laboratory to detect indirect alloreactivity; and Ref. 17). The studies confirm previously published work by a number of laboratories showing that human T cells can directly recognize and respond to xenantigens expressed on directly xenogeneic cells (1, 12, 13, 24, 26). Whether the post-transplant rejection response in humans is similarly mediated through direct recognition or is alternatively focused toward indirect reactivity remains to be established.

It is also intriguing that pork consumption was associated with a primed memory response to intact SLA (≤ peptide) rather than to indirectly presented swine Ags complexed to human HLA molecules across epithelial barriers (27, 28). Thus, it is theoretically possible under such a scenario that oral intake of pork could lead to an interaction of M cell-associated, gut-infiltrating lymphocytes with directly presented SLA molecules to prime an immune response. In conclusion, these studies provide the first evidence for the presence of primed T cell immunity toward swine Ags in adult human volunteers. The detected strength of this memory response raises the possibility that oral intake of pork could reactivate M cells in the gut that may present SLA to gut associated lymphocytes, leading to an interaction of M cell-associated, gut-infiltrating lymphocytes with directly presented SLA molecules to prime an immune response.

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