Anti-CD3/Anti-CD28 Bead Stimulation Overcomes CD3 Unresponsiveness in Patients With Head and Neck Squamous Cell Carcinoma

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Objectives: To test whether T-cell CD3 responses are altered in patients with advanced-stage head and neck squamous cell carcinoma (HNSCC) and whether anti-CD3/anti-CD28 (αCD3/αCD28) bead stimulation could reverse CD3 unresponsiveness.

Design: Anti-CD3 (αCD3) monoclonal antibody immobilized on tissue culture plastic was used to stimulate lymph node mononuclear cells (LNMCs) and peripheral blood mononuclear cells (PBMCs) from patients with advanced-stage HNSCC. Proliferation, T-cell phenotype, and cytokines were measured during 8-day in vitro stimulation. Immune-enhancing properties of αCD3/αCD28 beads were also tested on LNMCs and PBMCs. Cytotoxicity of bead-activated T cells (ATCs) was measured against autologous and allogeneic HNSCC.

Results: Six patients were nonresponders to αCD3 stimulation defined by tritium (3H) incorporation of less than 3500 cpm, whereas 11 patients were responders with 3H incorporation of 3500 cpm or more. Responders produced higher levels of interleukin (IL)–12 and interferon γ (IFN-γ) after αCD3 stimulation than nonresponders. No phenotypic or clinical differences were identified between groups. Stimulation with αCD3/αCD28 beads enhanced IFN-γ and IL-2 produced by both groups. Bead ATCs were generated from PBMCs of patient 11 in the responder group and lysed (± SD) 100% ± 1% of autologous tumor and 49% ± 1% of allogeneic tumor. Bead ATCs from LNMCs of this patient lysed 58% ± 1% of autologous tumor and 63% ± 1% of allogeneic tumor.

Conclusions: A subpopulation of patients with HNSCC who are nonresponders to αCD3 stimulation has been identified, showing reduced proliferation and IL-12 and IFN-γ secretion. Nonresponders stimulated with αCD3/αCD28 beads reversed immune unresponsiveness and induced a type 1 cytokine response. Bead-generated ATCs from patient 11 in the responder group lysed autologous and allogeneic HNSCC in vitro, suggesting a possible effective immunotherapeutic modality in the treatment of HNSCC.


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SUBJECTS AND METHODS

All individuals participating in our study provided informed consent, and a detailed explanation of the procedure, risks, and alternatives was given. The Human Investigation Committee and Institutional Review Board of Wayne State University, Detroit, Mich, granted approval for this study.

PATIENTS WITH HNSCC

Patients with advanced-stage HNSCC were studied because of the known immunosuppression that occurs with this cancer. Mean age of patients studied was 53.8 years, with a range of 38 to 68 years. All patients had advanced stage III (n = 5) or IV (n = 12) HNSCC. Primary sites of HNSCC included the oropharynx (n = 8), larynx (n = 5), oral cavity (n = 2), hypopharynx (n = 1), and an unknown primary site (n = 1).

PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood was drawn from patients before surgery. Blood was suspended in an equal volume of phosphate-buffered saline solution, and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque density gradient (Pel-Freez, Brown Deer, Wis) for 10 minutes at 2400 rpm.

LYMPH NODE MONONUCLEAR CELLS

Lymph nodes were harvested at the time of surgery and placed in balanced salt solution with 20% heat-inactivated fetal calf serum (Hyclone, Logan, Utah), a 1% combination of penicillin (Gibco, Paisley, Scotland) and streptomycin (Gibco), and 1% amphotericin B (Gibco). Lymph nodes were minced, filtered through a nylon mesh, and washed twice in balanced salt solution with 5% fetal calf serum, 1% penicillin-streptomycin, and 1% amphotericin B. Lymph node mononuclear cells (LNMCs) were obtained using Ficoll-Hypaque density gradient centrifugation described for PBMCs. Only pathologically confirmed negative lymph nodes were used in this study.

MoAb AND BEADS

Anti-CD3 (OKT3) MoAb was purchased from Caltag Corporation (Burlingame, Calif). The αCD3/αCD28 (9.3)–coated beads were provided by one of us (C.H.J.).

LYMPHOCYTE ACTIVATION

Lymphocytes were incubated in triplicate with uncoated plastic, αCD3-coated plastic, and αCD3/αCD28–coated bead for 8 days. The PBMCs (1.5 × 10⁶) were cultured in 200 µL of culture media in 96-well flat-bottom plates (Costar, Cambridge, Mass). Culture media consisted of RPMI 1640 (Gibco) supplemented with 10% fetal calf serum, 2-mlmoll/L glutamine (Gibco), penicillin (100 U/mL; Gibco), streptomycin (100 µg/mL; Gibco), and amphotericin B (100 U/mL; Gibco). For phenotyping and cytokine quantification, 7.5 × 10⁵ PBMCs or LNMCs were cultured in 2.0 mL of culture media in 24-well flat-bottom plates for 6 days. All cells were cultured at 37°C and 5% carbon dioxide.

PLASTIC STIMULATION

The 96- and 24-well microtiter plates were precoated with αCD3 at a concentration of 2 µg/mL in phosphate-buffered saline solution for 4 to 18 hours at 37°C and washed served in several malignant neoplasms. To test whether altered CD3 or TcR responses exist in patients with HNSCC, we stimulated LNL and PBL from patients with advanced-stage HNSCC with anti-CD3 (αCD3) monoclonal antibody (MoAb) and measured immune responses.

Several immunotherapeutic trials use αCD3 MoAb to stimulate T cells. The capacity of αCD3 to stimulate T cells is enhanced when combined with anti-CD28 (αCD28 or 9.3) MoAb, which substitutes for the B7.1 or B7.2 ligand and CD28 receptor in the absence of binding to cytotoxic T-lymphocyte antigen–4 (CD152), a negative regulator of the immune response. Anti-CD3/anti-CD28 (αCD3/αCD28) immobilized on iron beads has been used to overcome immunosuppression and generate bead-activated T cells (ATCs). Phase 1 adoptive immunotherapy trials using ATCs are being conducted in patients with human immunodeficiency virus (HIV), melanoma, and various solid tumors. In our study, αCD3/αCD28–coated beads were used to activate LNL and PBL from patients with advanced-stage HNSCC, and reversal of suppressed CD3 responses was measured.

RESULTS

T-CELL RESPONSES TO PLASTIC-ADHERENT αCD3

Anti-CD3 MoAb was immobilized on tissue culture plastic and used to stimulate LNMCs and PBMCs in vitro from patients with HNSCC. We chose CD3 because altered signaling via the TcR has been reported in other cancers. The optimal CD3-stimulating conditions were determined previously by proliferative responses of healthy control PBMCs. The LNMCs from 17 patients with advanced-stage cancer were stimulated with αCD3-coated plastic during an 8-day period. Eleven patients responded to αCD3 with [³H]-thymidine incorporation of 3500 cpm or more within the first 6 day of stimulation. They were defined as responders. Six patients did not respond to αCD3 and had [³H]-thymidine incorporation of less than 3500 cpm; they were defined as nonresponders (Figure 1). The difference in LNMC response to αCD3 stimulation between responders and non-
responders was significant (t test, P = .001 for day 4 and 
P = .002 for day 6) (Figure 1). Comparison of PBMC re-
sponse to αCD3 stimulation between responders and non-
responders was also significantly different (t test, P = .008 
for day 4 and P = .01 for day 6) (Figure 2).

CHARACTERIZATION OF RESPONDERS 
AND NONRESPONDERS

To identify differences between both groups in T-cell sub-
populations, lymph node and peripheral blood T cells were 
phenotyped before αCD3 stimulation. The percentage of 
CD3, CD4, CD8, CD28, and CD45RO T cells was compa-
rable in responders and nonresponders (Table 1). Shown in 
Table 1 is the phenotype of cells stimulated with αCD3/ 
αCD28 beads. Stimulation with αCD3 alone did not allow 
for adequate expansion of T cells for phenotypic analysis.

Cytokine expression from responders and nonre-
ponders was characterized and compared. Levels of IL-2, 
IL-4, IL-12, IFN-γ, and TNF-α were measured using ELISA 
on day 6. No difference was seen between groups in the 
cytokines spontaneously released by LNMCs (Table 2). 
Comparison of PBMCs revealed higher spontaneous 
expression of IL-12 from responders (P = .03, ANOVA). El-
evated IL-12 levels in unstimulated PBMCs from respond-

3 times with the phosphate-buffered saline solution. All 
αCD3 stimulation was performed with MoAb immobi-
лизирован. на основе культуры ткани.

BEAD STIMULATION

The αCD3/αCD28–coated bead stimulation was per-
formed at a 3:1 bead-lymphocyte ratio. For all samples, un-
stimulated controls were plated. Previous studies have 
shown that αCD28 alone and uncoated beads do not stimu-
late lymphocytes.20

[3H] THYMIDINE INCORPORATION BY PBMCs

On days 2, 4, 6, and 8, the cultures were pulsed with 
7.4 × 10^6 Bq of [3H] thymidine (tritium thymidine) for 4 
hours and harvested onto glass fiber discs using a cell har-
vester (PhD; Cambridge Technology, Cambridge, Mass). 
The glass fiber discs were counted in vials containing 6 mL 
of scintillation-counting cocktail in a scintillation counter 
(Beckman, Fullerton, Calif).

PHENOTYPIC ANALYSIS

On days 0 and 6 after incubation, cell suspensions were pre-
pared from LNMC or PBMC cultures and stained with MoAb 
to CD3, CD4, CD8, CD28, or CD45RO conjugated to phy-
coerythrin (PharMingen, San Diego, Calif) as previously de-
described.21 Surface marker expression was measured by flow 
cytometry (FACScan; Becton Dickinson, San Jose, Calif).

CYTOKINE ANALYSIS

Cell culture supernatants were harvested on days 2, 4, 6, 
and 8. The quantity of IL-2, IL-4, TNF-α, IL-12, and IFN-γ 
present in the supernatant was determined using an enzyme-
linked immunosorbent assay (ELISA) (R & D Systems, Min-
neapolis, Minn).

CYTOKINE ANALYSIS

Stimulation of LNMCs with αCD3-coated plastic en-
Riched the production of IFN-γ (P = .05, ANOVA) and 
IL-12 (P = .05, ANOVA) in responders but not nonre-
ponders (Table 2). Stimulation of PBMCs also enhanced 
IL-12 (P = .03, ANOVA) in responders (Table 2). Respond-
ers produced more IFN-γ after αCD3 stimulation than did 
nonresponders. Also important to note is that the levels of 
cytokines released were much higher after αCD3/αCD28 
stimulation than after αCD3 stimulation alone (Table 2).

αCD3/αCD28 BEAD COS!TMULATION 
OF NONRESPONDERS AND RESPONDERS

Because of the poor proliferation and IFN-γ production in 
nonresponders, we performed αCD3/αCD28 bead stimu-
lation to test whether immune unresponsiveness 
could be reversed. Costimulation with αCD3/αCD28 
beads is presently used in several adoptive immunothera-
peutic trials to enhance immune function against infec-
tion and cancer. The responders functioned as a com-
parison group for nonresponders. Proliferative responses 
were measured over an 8-day period during bead stimu-
lations. Significantly higher proliferation continued to oc-
cur in LNMCs on day 4 in responders compared with non-
responders (P = .02, t test) (Figure 3). Differences 
between responders and nonresponders on day 6 were
Peripheral Blood

Table 1. Phenotype of T Cells From Lymph Node and Peripheral Blood*

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Lymph Node</th>
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<th>Peripheral Blood</th>
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<tr>
<td></td>
<td>Responders</td>
<td>Nonresponders</td>
<td>Responders</td>
<td>Nonresponders</td>
<td>Responders</td>
<td>Nonresponders</td>
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<tr>
<td>On Day 0†</td>
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</tr>
<tr>
<td>CD3</td>
<td>46 ± 21</td>
<td>52 ± 19</td>
<td>63 ± 21</td>
<td>58 ± 23</td>
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<tr>
<td>CD4</td>
<td>27 ± 13</td>
<td>36 ± 12</td>
<td>40 ± 16</td>
<td>33 ± 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>11 ± 7</td>
<td>17 ± 10</td>
<td>22 ± 12</td>
<td>23 ± 12</td>
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<td></td>
</tr>
<tr>
<td>CD28</td>
<td>39 ± 13</td>
<td>48 ± 21‡</td>
<td>51 ± 25</td>
<td>54 ± 21‡</td>
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<tr>
<td>CD45RO</td>
<td>19 ± 13§</td>
<td>27 ± 17</td>
<td>18 ± 7§</td>
<td>23 ± 11</td>
<td></td>
<td></td>
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<tr>
<td>On Day 6‡</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD3</td>
<td>66 ± 22</td>
<td>95 ± 49§</td>
<td>84 ± 6</td>
<td>95 ± 6‡</td>
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<td>CD4</td>
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<td>64 ± 11</td>
<td>61 ± 11</td>
<td>72 ± 9</td>
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<td>CD8</td>
<td>19 ± 6</td>
<td>21 ± 5</td>
<td>19 ± 11</td>
<td>21 ± 4</td>
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<td></td>
</tr>
<tr>
<td>CD28</td>
<td>66 ± 29</td>
<td>92 ± 6#</td>
<td>80 ± 7</td>
<td>85 ± 19#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RO</td>
<td>54 ± 11**</td>
<td>73 ± 14</td>
<td>73 ± 10§</td>
<td>82 ± 22</td>
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</tr>
</tbody>
</table>

* Data are given as mean ± SD percentage.
† For responders, n = 11; for nonresponders, n = 5, unless specified.
‡ N = 4.
§ N = 8.
¶ After anti-CD3/anti-CD28 bead stimulation. For responders, n = 10; for nonresponders, n = 3, unless specified.
* Significant difference between responders and nonresponders (P < .05, t test).
# N = 2.
** N = 6.

Figure 1. Anti-CD3 stimulation of lymph node mononuclear cells. Proliferative responses were significantly higher in responders (P = .001 for day 4; P = .002 for day 6). *H indicates tritium.

Figure 2. Anti-CD3 stimulation of peripheral blood mononuclear cells. Proliferative responses were significantly higher in responders (P = .008 for day 4; P = .01 for day 6). *H indicates tritium.

reduced to insignificant levels (Figure 3). Differences between responders and nonresponders in PBMC proliferation on day 4 and 6 were reduced to insignificant levels (Figure 4). Proliferative deficiency of nonresponders to αCD3 stimulation was partially reversed by αCD3/αCD28 bead stimulation.

To identify differences in T-cell subpopulations between groups, lymph node T cells were phenotyped after αCD3/αCD28 bead stimulation. Expression of T-cell CD3, CD4, CD8, CD28, and CD45RO from responders and nonresponders was measured and compared on day 6. Similarly, peripheral blood T cells were phenotyped after αCD3/αCD28 bead stimulation and compared on day 6. There was a significantly higher percentage of CD3+ cells in nonresponder lymph node and peripheral blood T cells after αCD3/αCD28 stimulation compared with responders (Table 1). No other differences were identified.

To compare the immunologic environment existing in responders and nonresponders after αCD3/αCD28 bead stimulation, LNMCs and PBMCs were stimulated in vitro, and the production of IL-2, IL-4, IL-12, IFN-γ, and TNF-α was measured on day 6 (Table 2). There were no significant differences in cytokines produced by responders and nonresponders. Bead stimulation enhanced IL-2, IFN-γ, and TNF-α secretion in both groups from LNMCs as well as PBMCs (Table 2). The previously observed differences in IL-12 and IFN-γ levels were overcome, and a strong Th1 cytokine profile was generated in responders and nonresponders.

αCD3/αCD28 BEAD VS αCD3-COATED PLASTIC STIMULATION

Stimulation with αCD3/αCD28 beads was compared with αCD3 plastic stimulation for efficacy in inducing a Th1 immune response characterized by high levels of IL-2 and IFN-γ secretion. A Th1 immune response is highly desirable in fighting cancer. Bead stimulation significantly enhanced levels of IL-2 and IFN-γ in responders (IL-2, P = .001, t test; IFN-γ, P = .005) and IL-2 in nonresponders (P = .05) compared with αCD3 plastic stimulation (Table 2). Stimulation of αCD3/αCD28 beads was more effective than that of αCD3-coated plastic in producing TNF-α in responders (P = .005, t test) (Table 2).

CYTOTOXICITY OF αCD3/αCD28 ATCs

To further investigate the immune-enhancing properties of αCD3/αCD28 bead stimulation, the cytotoxicity of bead-generated ATCs was measured against autologous tumor. Patient 11 from the responders was chosen because tumor had been successfully cultured, and sufficient numbers of LNMCs and PBMCs were available for study. The ATCs were generated from responder 11 by stimulating LNMCs and PBMCs for 6 days in vitro with coated beads. Tumor cell lysis was measured using an 18-hour 51Cr release assay with effector-target ratios of 50:1, 25:1, and 12:1, respectively. Lymph node ATCs showed lysis (±SD) of 55% ± 1%, 58% ± 1%, and 44% ± 1%, respectively (Figure 5). Peripheral blood ATCs

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showed lysis (±SD) of 100% ± 1%, 100% ± 1%, and 89% ± 1%, respectively (Figure 5). Unstimulated lymph node and peripheral blood T cells showed minimal lysis (range, 0%-4%) (Figure 5). A 4-hour 51Cr release assay was also performed, which showed minimal lysis (range, 0%-9%). Supernatant from bead-activated peripheral blood and lymph node ATCs was tested against autologous tumor and showed minimal lysis (range, 0%-10%).

Further testing of ATC from responder 11 against allogeneic tumor was performed. Lysis was measured using an 18-hour 51Cr release assay with effector-target ratios of 25:1, 12:1, 6:1, and 3:1, respectively. Lymph node ATCs showed lysis (±SD) of 63% ± 1%, 58% ± 1%, 40% ± 1%, and 22% ± 1%, respectively (Figure 6). Peripheral blood ATCs showed lysis (±SD) of 49% ± 1%, 40% ± 1%, 27% ± 1%, and 23% ± 1%, respectively (Figure 6). Unstimulated lymph node and peripheral blood ATCs showed minimal lysis (range, 0%-2%). Supernatant from bead-activated peripheral blood and lymph node ATCs was tested against allogeneic tumor and showed minimal lysis (range, 0%-10%). Lysis of allogeneic tumor showed that killing was partially nonspecific in nature.

### Table 2. Cytokines Secreted on Day 6 by Responders and Nonresponders*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LNMC, pg/mL</th>
<th>PBMC, pg/mL</th>
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<tbody>
<tr>
<td></td>
<td>Responders</td>
<td>Nonresponders</td>
</tr>
<tr>
<td></td>
<td>Unstimulated</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>0 ± 0</td>
<td>2 ± 4</td>
</tr>
<tr>
<td>IL-4</td>
<td>81 ± 112</td>
<td>3 ± 5</td>
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<tr>
<td>IL-12</td>
<td>1 ± 3</td>
<td>3 ± 5</td>
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<tr>
<td>TNF-α</td>
<td>50 ± 113</td>
<td>122 ± 147†</td>
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<td></td>
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<tr>
<td></td>
<td>After αCD3 Plastic Stimulation</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>6 ± 7†</td>
<td>17 ± 16‖</td>
</tr>
<tr>
<td>IL-4</td>
<td>48 ± 91‖</td>
<td>138 ± 188‖</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1 ± 29‡</td>
<td>14 ± 17‖</td>
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<tr>
<td>TNF-α</td>
<td>31 ± 86‖</td>
<td>0 ± 0</td>
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<tr>
<td></td>
<td>After αCD3/αCD28 Bead Stimulation</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>2153 ± 1309***</td>
<td>1481 ± 1215***</td>
</tr>
<tr>
<td>IL-4</td>
<td>21 ± 19</td>
<td>33 ± 27</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1677 ± 851**</td>
<td>1074 ± 872**</td>
</tr>
<tr>
<td>IL-12</td>
<td>35 ± 39</td>
<td>170 ± 181</td>
</tr>
<tr>
<td>TNF-α</td>
<td>293 ± 298**</td>
<td>584 ± 420**</td>
</tr>
</tbody>
</table>

*LNMC indicates lymph node mononuclear cells; PBMC, peripheral blood mononuclear cells; IL, interleukin; INF-γ, interferon-γ; TNF-α, tumor necrosis factor-α; αCD3, anti-CD3 monoclonal antibody; and αCD28, anti-CD28 monoclonal antibody. Data are given as mean ± SD. Unless otherwise specified, n = 10 for responders; n = 6 for nonresponders.

†P < .05, t test, responders vs nonresponders.
‡N = 8.
§N = 3.
‖N = 4.
¶N = 2.
#N = 9.
**P < .05, t test, αCD3/αCD28 bead-stimulated vs αCD3-stimulated group.
Recent studies from several laboratories suggest that alterations in function and expression of signal-transducing molecules associated with CD3 are responsible for the immune deficiencies observed in malignant melanoma and renal cell, ovarian, and colorectal carcinoma.5-8 To test whether altered CD3 or TcR responses exist in patients with HNSCC, we stimulated LNLs and PBLs from patients with advanced-stage cancer with αCD3 MoAb and measured immune responses. By this method, 2 populations of patients, responders and nonresponders based on LNL (Figure 1) and PBL responses (Figure 2), were identified. The presence of CD3 on the surface of LNLs and PBLs was checked and confirmed by flow cytometry (Table 1). Flow cytometric analysis revealed no differences in T-cell subpopulations between groups (Table 2), and there was no clinical difference between groups. Responders included 3 patients with stage III and 8 patients with stage IV HNSCC. Nonresponders included 2 patients with stage III and 4 patients with stage IV disease. There was no difference in tumor burden between groups. Further study will be required to identify the specific defect in CD3 response observed herein. Others have attributed altered CD3 responses to loss of CD3-ζ chain expression.5-8

The characterization of responders revealed a difference in the spontaneous release of cytokines compared with nonresponders. A higher level of IL-12 was produced by PBMCs in responders compared with nonresponders (Table 2). Elevated IL-12 levels suggested a baseline T H1 immune environment existed in the responders (Table 2). It may be possible that APCs or B cells in responders secret higher levels of IL-12, predisposing this subset of patients to a T H1 response.27 There were no treatment difference between responders and nonresponders; all had advanced-stage HNSCC and had not previously received chemotherapy or radiation therapy. The LNLs and PBMCs cultured in these experiments were a mixture of T and B cells. Flow cytometry before culturing showed no significant difference in T-cell populations between groups (Table 1). Higher baseline levels of IL-12 in the immune environment correspond to enhanced production of IFN-γ during immune stimulation.23,24 No differences were identified in baseline levels of IL-4, a T H2 type of cytokine; expression of IL-10 and tumor growth factor β was not checked.

Stimulation of both groups with αCD3 revealed that responders produced higher levels of IL-12 and IFN-γ compared with nonresponders (Table 2). This demonstrated that the immune environment of responders favors a T H1 response, whereas nonresponders showed minimal response. Reduced response to αCD3 stimulation may be caused by alterations in the CD3-ζ chain function or the presence of immunosuppressive factors such as prostaglandin E2 or tumor growth factor β.25,26 The exact cause of immune unresponsiveness has not been identified.

To test whether αCD3/αCD28 bead stimulation can overcome the suppressed responses identified in nonresponders, we stimulated both groups and compared results. Stimulation with αCD3/αCD28 beads was chosen because this method of T-cell stimulation is being studied in adoptive immunotherapeutic trials for the treatment of HIV, melanoma, lymphoma, and various solid tumors.17-20 We have reported previously on the immune-enhancing properties of αCD3/αCD28–coated surgical suture.27 Stimulation with αCD3/αCD28 beads enhanced proliferation in both groups (Figures 3 and 4). Bead stimulation enhanced the production of IL-2, IFN-γ, and TNF-α in both groups (Table 2). Differences between responders and nonresponders in secretion of cytokines—spontaneously or after αCD3 stimulation—were reversed with αCD3/αCD28 bead stimulation. Bead stimulation generated more T H1 cytokine production in both groups (Table 2).

The cytotoxicity of bead-stimulated ATCs against autologous cancer was tested. Peripheral blood ATCs from responder 11 showed 100% ± 1% lysis of tumor at 50:1 and 25:1 effector-target ratios, with 89% ± 1% lysis at 12:1. Lymph node ATCs killed in the range of 50% to 60% (± 1%), with effector-target ratios of 50:1, 25:1, and 12:1, respectively. The specificity of kill was measured by using allogeneic HNSCC as targets. Peripheral blood and lymph node ATCs showed lysis of 23% to 49% (± 1%) and 22% to 63% (± 1%), respectively. Therefore, a por-
tion of tumor kill was nonspecific in nature. To test whether lysis resulted from a soluble factor, supernatant was tested against autologous and allogeneic tumor. Minimal lysis (0%-10%) was noted, indicating the absence of a soluble cytolytic factor. Cytotoxicity assays were performed for 4 and 18 hours. Four-hour incubations showed minimal lysis (0%-4%), whereas 18-hour incubations increased kill up to 100%. The significance of prolonging incubations is not clear. Cytotoxicity experiments were performed in only 1 patient (responder 11), because the availability of autologous tumor, LNMCs, and PBMCs was limiting. Cells from other individuals may have a different response.

The generation of a T_{H}1 immune response makes this method of T-cell stimulation an attractive immunotherapeutic option for treating HNSCC. Cytokines showing T_{H}1 have been associated with cytotoxic immune responses against cancer. In fact, our findings are consistent with those of other investigators who have found enhanced immune function with αCD3/αCD28 bead stimulation of cells from patients seropositive for HIV. 19 In a phase 1 trial involving autologous reinfusion of CD4 T cells to patients who are seropositive for HIV, a long-term rise in CD4 counts has been observed (C.H.J., personal communication, May 1999). The in vitro human data presented in our study strongly support the development of a phase 1 adoptive immunotherapy trial to test αCD3/αCD28 bead-generated ATCs against advanced-stage HNSCC.

Subpopulations of patients with HNSCC have been identified who are nonresponders to αCD3 stimulation. Nonresponders showed reduced expression of IL-12 and IFN-γ compared with responders. Stimulation with αCD3/αCD28 beads overcame the CD3 unresponsiveness identified in nonresponders and generated a T_{H}1 immune response. The αCD3/αCD28 bead-generated ATCs from responder 11 were able to lyse autologous and allogeneic HNSCC in vitro. Costimulation with αCD3/αCD28 beads generated ATCs that may be an effective immunotherapeutic modality in the treatment of HNSCC.

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