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Potent Costimulation of Effector T Lymphocytes by Human Collagen Type I

Wei Hong Rao, Jonathan M. Hales, and Richard D. R. Camp

Purified, resting peripheral blood T lymphocytes were previously reported to undergo $\beta_1$ integrin-dependent activation when cultured with anti-CD3 mAb coimmobilized with fibronectin, but not type I collagen. However, the extravascular T cells that encounter immobilized extracellular matrix proteins and are involved in disease pathogenesis have different properties from resting peripheral blood cells. In this study, we confirm that resting CD4$^+$ and CD8$^+$ T cells from peripheral blood are costimulated by immobilized fibronectin, but not type I collagen. In contrast, Ag- or mitogen-stimulated CD4$^+$ and CD8$^+$ T cell lines, used as models of the effector cells involved in disease, are more potently costimulated by type I collagen than fibronectin. The collagen-induced effects are similar in assays with serum-free medium and in more physiological assays in which anti-CD3 mAb is replaced by a threshold concentration of Ag and irradiated autologous PBMC as APC. The responses are $\beta_1$ integrin dependent and mediated largely by very late Ag (VLA) 1 and 2, as shown by their up-regulation on the T cell lines as compared with freshly purified resting PBL, and by the effects of blocking mAb. Reversed phase HPLC located the major costimulatory sequence(s) in the $\alpha_1$ chain of type I collagen, the structure of which was confirmed by amino acid sequencing. The results demonstrate the potential importance of type I collagen, an abundant extracellular matrix protein, in enhancing the activation of extravascular effector T cells in inflammatory disease, and point to a new immunotherapeutic target.


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3 Abbreviations used in this paper: ECMP, extracellular matrix protein; VLA, very late Ag..
of the extravascular effector cells encountered in disease. We have confirmed that purified, resting peripheral blood T cells are co-
stimulated by immobilized fibronectin and laminin, but not type I
collagen. In contrast, we have shown that immobilized human type
I collagen is a highly potent, $\beta_1$ integrin-dependent costimulator of
the effector T cell populations, with a potency greater than that of
fibronectin in the previously reported bioassays (4, 5) incorporat-
ing coimmobilized anti-CD3 mAb.

Materials and Methods

Abs and reagents

Fibronectin from human plasma, laminin from human placenta, collagen
types I and III from human placenta, PHA, and Con A were purchased
from Sigma (Poole, U.K.). Stock solutions of the collagens were made by
redissolving the lyophilized preparations in 0.5 M acetic acid at 1 mg/ml,
and were stored at 4°C. Fibronectin was supplied as a lyophilized of 0.05
M TBS. Stock solution was prepared by diluting this to 1 mg/ml in water,
and stored in aliquots at −20°C. Laminin was supplied in Tris-buffered
NaCl at 0.5 mg/ml and was stored at −80°C in aliquots. These storage
conditions were as recommended by the supplier. CD3 mAb OKT3 was
from Clig Biotech (High Wycombe, U.K.), and CD29 mAb 4B4 from
Beckman Coulter (High Wycombe, U.K.). Purified mAbs against $\alpha_5$ int-
egrins (clones FB12, P166, ASC-1, P1H4, and P1D6, respectively), used
mainly for flow cytometry, were from Chemicon (Harrow, U.K.); FITC-
conjugated mouse anti-human CD4 and CD8 mAb and negative control
Ab from Dako (Ely, U.K.); and PE-conjugated goat anti-mouse IgG from Se-
rotec (Oxford, U.K.). Purified IgG1-blocking mAb AJH10 and 26F8,
against $\alpha_1$ and $\alpha_2$ integrins, respectively, were obtained from Dr. Roy Lobb
(Biogen, Cambridge, MA). Blocking mAb against $\alpha_4$ (P1B5; IgG1) and $\alpha_5$
(P4G9; IgG3) integrins, both in dialyzed culture supernatant, were from
Dako. Blocking mAb against $\alpha_3$ integrin (purified SAM-1; IgG2b) was
from Serotec. Purified, isotype control IgG3 and IgG2b mAb were from
Dako and IgG1 was a gift from Dr. R. James, University of Leicester
(Leicester, U.K.). Tetanus toxoid was from Evans (Horsham, U.K.),
and rIL-2 from Eurocetus (Harefield, U.K.).

Preparation of fresh, resting human peripheral blood CD4+ and
CD8+ T cells

PBMC were obtained from citrate anticoagulated blood of healthy adult
volunteers by standard density-gradient centrifugation over Lymphoprep
(Life Technologies, Paisley, U.K.). Interface PBMC were pelleted, washed,
and resuspended in Earle’s balanced salt solution at 4°C. For purification
of CD4+ T cells, PBMC were resuspended in 1 ml Earle’s balanced salt
solution with Dynabeads M-450 CD4 (Dynal, Bromborough, U.K.) at
a ratio of beads to target cells of 4:1. Dynabead-bound T cells were separated
and thoroughly washed in accordance with the manufacturer’s instructions,
then resuspended in 100 μl RPMI 1640 medium containing 10% pooled
human AB serum, 3 mM l-glutamine, 100 μM penicillin, and 100 μg/ml strepto-
inulin. After 10–20 min, the supernatant was removed and the remaining
ells were then used in one of the following experiments. For the
immobilized anti-CD3 protocol, the cells were resuspended at
a 1:2 ratio in RPMI supplemented with 10% FCS and cultured
for 7 days with immobilized anti-CD3 (0.1 μg/ml), and IL-2 as
supplemented with fresh medium and pulsed with IL-2 (100 U/ml)
every 3–4 days. Following the last round of Ag stimulation, cells were pulsed with
2 μCi/well [3H]TdR, T cells were harvested, and incorporated radioactivity
was determined. In certain proliferation assays, freshly purified CD4+ T cells
from peripheral blood or tetanus toxoid-responsive CD4+ T cell lines (5
× 105) were incubated with twice the number of irradiated, autologous
PBMC, and appropriate concentrations of tetanus toxoid in ECMP-coated
or uncoated microtiter plate wells for 3–5 days, and [3H]TdR uptake de-
termined. In other experiments, mAb that blocked integrin function (up to
10 μg/ml), and isotopes were added to 3-day proliferation assays
with T cell lines, coimmobilized anti-CD3 (0.1 μg/ml), and either fibronectin
(10 μg/ml) or type I collagen (1 μg/ml). In experiments to test the
effects of the $\beta_1$ integrin-specific mAb 4B4 in ECMP-free assays, a CD8+
T cell line was cultured in the presence of 50–200 U/ml IL-2 and up to 2 mg/ml
of type I collagen in wells coated with anti-CD3 (0.1 μg/ml), and
[3H]TdR uptake determined. Results from all T cell proliferation as-
says are expressed as mean cpm from triplicate cultures.

HPLC, SDS-PAGE, trypsin digestion, and amino acid
sequencing of type I collagen

Stock solutions of type I collagen were purified in 100-μg aliquots by
sequential reversed phase HPLC on a 4.6 × 100-mm Brownlee Aquapore
RP-300 C8 column and a 4.6 × 250-mm Atlantis 5 μ C18 300 Å column
(Phenomenex, Macclesfield, U.K.), both eluted at 1 ml/min with 0.1%
trifluoroacetic acid in water for 5 min, followed by a linear gradient to
0.1% trifluoroacetic acid in acetonitrile over 30 min. UV-absorbing peaks
(215 nm) were collected manually, evaporated, and tested for T cell co-
stimulatory activity. Aliquots of purified peptides were analyzed by SDS-
PAGE on 6% acrylamide minigels. Following staining with Coomassie
blue or 100-kDa band corresponding to the α1 chain of type I collagen
was excised and trypsinized by methods established in the University of
Leicester Protein and Nucleic Acid Chemistry Laboratory. Briefly, the
band was destained repeatedly in ethanol/50 mM ammonium bicarbonate
(2:3, v/v), dehydrated by washing in acetonitrile followed by vacuum des-
ication, rehydrated in 10 mM DTT/100 mM ammonium bicarbonate, de-
hydrated once again, and digested by incubation with trypsin (75 μg/ml in
0.1% trifluoroacetic acid in water, 1:500, for 3 h). The resulting peptides
were extracted from the gel into 5% trifluoroacetic acid in acetonitrile/water
(1:1, v/v), and following evaporation the residue was purified on a
1 × 250-mm C18 Aquapore RP-300 HPLC column eluted with an aceto-
nitrile gradient. Residue from a peak eluting at ~25% acetonitrile was
subjected to N-terminal amino acid sequencing in an ABI 476 liquid-phase
protein sequencer (Applied Biosystems, Foster City, CA).

Results

Effect of coimmobilized ECMP and anti-CD3 mAb on
proliferation of freshly purified, resting peripheral blood T cells, and
T cell lines

Freshly purified, unstimulated peripheral blood CD4+ and CD8+ T cells
did not proliferate in the presence of immobilized anti-CD3 or
ECMP alone (data not shown), but underwent substantial pro-
liferation when cultured in microtiter wells coated with anti-CD3
and fibronectin, as found previously (3–5). Proliferation was less
pronounced with laminin coating, and only minimal or absent with
collagen types 1 or 3 (Fig. 1, A and B), thus confirming two
previous reports (4, 5). In contrast, CD4+ and CD8+ T cell lines
proliferated strongly in the presence of coimmobilized anti-CD3 and
collagen type I, with the effects of fibronectin, laminin, and
collagen type III apparently being less potent that those of type I

Flow cytometric analysis

T cells (5 × 104 in 100 μl PBS containing 2% BSA) were incubated with
$\beta_1$, or a integrin mAb, IgG1, or IgG3 isotype controls (all 2 μg/ml), fol-
lowed by PE-conjugated goat anti-mouse IgG (1/10 final dilution). Cells
were also labeled with FITC-conjugated mouse anti-human CD4 or CD8 mAb
(10 μg/ml) before analysis by FACScan (Becton Dickinson, Oxford, U.K.).

T cell proliferation assays

Anti-CD3 mAb OKT3 (0.5 μg/ml in PBS for experiments with fresh, rest-
ning PBL as in Fig. 1, A and B, and 0.1 μg/ml for all other experiments) was
placed in microtiter plate wells and incubated overnight at 4°C. After three
washes with PBS to remove unbound mAb, different amounts of ECMP
were added for 2–3 h at room temperature (4). Unbound ECMP was re-
moved by three washes with PBS. T cells (5 × 105/well) were then added in
RPMI or AIM V serum-free medium (Life Technologies, Gaithersburg,
MD), and incubated for 3 days. During the last 8 h of the assay, cultures were
pulsed with 1 μCi/well [3H]TdR. T cells were harvested, and incorporated radioactivity
was counted. In certain proliferation assays, freshly purified CD4+ T cells
from peripheral blood or tetanus toxoid-responsive CD4+ T cell lines (5
× 105) were incubated with twice the number of irradiated, autologous
PBMC, and appropriate concentrations of tetanus toxoid in ECMP-coated
or uncoated microtiter plate wells for 3–5 days, and [3H]TdR uptake de-
termined. In other experiments, mAb that blocked integrin function (up to
10 μg/ml), and isotopes were added to 3-day proliferation assays
with T cell lines, coimmobilized anti-CD3 (0.1 μg/ml), and either fibronectin
(10 μg/ml) or type I collagen (1 μg/ml). In experiments to test the
effects of the $\beta_1$ integrin-specific mAb 4B4 in ECMP-free assays, a CD8+
T cell line was cultured in the presence of 50–200 U/ml IL-2 and up to 2 mg/ml
type I collagen in wells coated with anti-CD3 (0.1 μg/ml), and
[3H]TdR uptake determined. Results from all T cell proliferation as-
says are expressed as mean cpm from triplicate cultures.
collagen on a μg/ml basis. Thus, a low coating concentration of type I collagen (0.6 μg/ml) caused marked CD4+ and CD8+ T cell stimulation, whereas the same coating concentration of fibronectin and laminin gave small responses. Collagen type III gave intermediate responses at this concentration (Fig. 1, C and D). The greater potency of collagen type I than fibronectin in costimulating a CD8+ T cell line was demonstrated in further assays incorporating immobilized anti-CD3 and a wider range of concentrations of fibronectin and collagen type I. In these assays, the concentrations of collagen type I and fibronectin causing half-maximal responses were ~0.04 μg/ml and at least 2.5 μg/ml, respectively, indicating at least 60-fold greater potency for collagen type I in this system, on a μg/ml basis (Fig. 1E). The higher concentrations of type I collagen were associated with apparently decreasing responses, as also seen in Fig. 1, C and D. The mechanism(s) responsible for this bell-shaped dose-response curve has not yet been investigated. Activation of T cell lines by the collagen type I was also shown to be independent of serum factors such as fibronectin, as T cell proliferation was not reduced in costimulation assays with AIM V serum-free medium vs RH10 (Fig. 2, A and B).

Effect of immobilized type I collagen and fibronectin on Ag-induced T cell proliferation

Preliminary experiments determined the threshold concentrations of tetanus toxoid that caused proliferation of either freshly purified peripheral blood CD4+ T cells, or CD4+ T cell lines, to levels just above background in 5- or 3-day assays, respectively, with autologous, irradiated PBMC as APC (data not shown). Coating of microtiter wells with type I collagen or fibronectin was subsequently shown to cause marked enhancement of proliferation of the CD4+ line cultured with irradiated PBMC and the threshold amount of tetanus toxoid, coating concentrations of ECMP as low as 20 ng/ml, causing maximal costimulation (Fig. 3B). In this system, the μg/ml potencies of type I collagen and fibronectin were similar. In contrast and in line with results obtained in initial experiments with immobilized anti-CD3 (Fig. 1A), 5-day assays with freshly purified peripheral blood CD4+ T cells, irradiated autologous PBMC, and a threshold concentration of tetanus toxoid showed enhancement of proliferation by fibronectin, but not type I collagen (Fig. 3A).

Enhanced expression of α1, α2, and α3 integrins on T cell lines, and integrin dependence of type I collagen-induced responses

Two-color flow cytometry was used to determine the percentage of T cells expressing specific integrins (Table I). As shown, the percentages of resting, freshly purified peripheral blood CD4+ cells expressing α1, α2, and α3 integrins were generally low, but expression of these integrins by the corresponding T cell line from the same donor was greatly enhanced. A similar trend was seen with CD8+ cells. The percentage of expression of α1 and β1 integrins was high for both the resting peripheral blood cells and the T cell lines. The percentage of expression of α5 integrin by the

FIGURE 1. Effects of coimmobilized anti-CD3 and ECMP on proliferation of T cells cultured in medium containing 10% human serum. Proliferation of resting, freshly harvested CD4+ and CD8+ peripheral blood T cells is shown in A and B, respectively, in which coating concentrations of ECMP are 2 μg/ml (■) and 10 μg/ml (□). Proliferation of CD4+ and CD8+ T cell lines is shown in C and D, respectively, in which the coating concentrations of ECMP in the different groups of columns are, from left to right, 0.6 μg/ml, 1.25 μg/ml, 2.5 μg/ml, 5 μg/ml (C and D), and in addition 10 and 20 μg/ml (C). Similar results to those in A–D were obtained in at least one more set of independent triplicate experiments. FN, Fibronectin; LN, laminin; COL I, type I collagen; COL III, type III collagen. In E, the effects of a wider range of coating concentrations of type I collagen (□) and fibronectin (△) on the proliferation of a CD8+ T cell line are shown. All points represent the mean results of triplicate cultures.

FIGURE 2. Effects of coimmobilized anti-CD3 and type I collagen (A) or fibronectin (B) on proliferation of a CD4+ T cell line cultured in serum-free medium (■) or RH10 (△). Data are the mean results of triplicate cultures. Similar results were obtained in a second, independent, triplicate experiment. The responses of a CD8+ T cell line to coimmobilized anti-CD3 and type I collagen or fibronectin were also at least as great in serum-free medium as in RH10 (data not shown). Abbreviations for ECMP are as in Fig. 1.
freshly purified, resting cells was lower than for \( \alpha_3 \), but was enhanced on the T cell lines. The previously reported bimodal expression of \( \beta_1 \) integrin on freshly harvested, resting CD4\(^+\) T cells (4, 31) was confirmed in the present experiments, being less pronounced on the resting CD8\(^+\) cells, but was seen on both CD4\(^+\) and CD8\(^+\) T cell lines (data not shown).

The proliferative responses of T cell lines cultured with coimmobilized anti-CD3 and type I collagen were blocked by the \( \beta_1 \) integrin-blocking mAb 4B4, concentrations as low as 32 ng/ml having a major inhibitory effect (Fig. 4, A and B). That effect of 4B4 mAb was not due to nonspecific cytotoxicity or negative signaling via \( \beta_1 \) integrin was shown by the lack of any inhibitory effect of 4B4 on a CD8\(^+\) T cell line proliferating in ECMP-free conditions, in the presence of immobilized anti-CD3 and three different concentrations of IL-2 (Fig. 4C).

The dependence of type I collagen-induced T cell costimulation on VLA integrin subtype was explored further by using \( \alpha \) integrin-specific blocking mAb. As previously reported (4, 5), fibronectin-induced costimulation of T cells was reduced by mAb against VLA-4 or VLA-5 integrin (mAb P4G9 or SAM-1, respectively, in the present experiments), and completely blocked by a combina-

tion of the two (Fig. 4D). In contrast, type I collagen-induced costimulation of a CD8\(^+\) T cell line was unaffected by VLA-4 or VLA-5 mAb or a combination of these, but was partially blocked by VLA-1 or VLA-2 mAb (AJH10 or 26F8, respectively), with more pronounced inhibition by a combination of the two mAbs (Fig. 4E). The VLA-3 mAb P1B5 (Dako) inhibited both fibronectin- and type I collagen-induced T cell costimulation (data not shown), suggesting uncertain integrin-blocking specificity. Similarly, the VLA-3 mAb P1B5 (Chemicon) is reported by the manufacturers not to inhibit cell adhesion to fibronectin. However, we found that it inhibited both fibronectin- and type I collagen-induced T cell costimulation (data not shown), suggesting uncertain integrin-blocking specificity. Evidence for VLA-3 involvement in the collagen-induced responses has therefore not been established.

### Purification and structural analysis of type I collagen

The type I collagen preparation (Sigma) used in this work was prepared by acidic pepsin digestion of human placental homograft, and differential salt precipitation (32). Reversed phase HPLC of this preparation on a 4.6 × 100-mm C8 column, under the conditions described in Materials and Methods, demonstrated a product of substantial purity (peaks 1 and 2, Fig. 5A). Repurification of each peak on a 4.6 × 250-mm C18 column yielded separated peaks (1a and 2a, Fig. 5A). Following Micro bicinechonic acid protein assay (Pierce and Warriner, Chester, U.K.), the ability of equivalent amounts of protein from each peak to stimulate a T cell line in the presence of coimmobilized anti-CD3 was determined. This showed that peak 1a contained the major stimulatory material (Fig. 5C). SDS-PAGE of the unpurified type I collagen preparation showed a major Coomassie blue-stained band at about 100 kDa and a less intense band at about 95 kDa, compatible with the \( \alpha_1 \) and \( \alpha_2 \) chains of type I collagen, which are present at a ratio of 2:1 (33). Higher molecular mass bands were also present, suggestive of dimeric and trimeric forms (Fig. 5B, lane 1). SDS-PAGE of peak 1a (Fig. 5A) showed a single, major Coomassie blue-stained band at ~100 kDa, indicative of the \( \alpha_1 \) chain of type I collagen, and less prominent higher molecular mass bands (Fig. 5B, lane 2). SDS-PAGE of protein from peak 2a (Fig. 5A) showed a major band at ~95 kDa, indicative of the \( \alpha_2 \) chain of type I collagen, with a second, fainter band at ~100 kDa indicating carry-over from peak 1, and prominent higher molecular mass bands (Fig. 5B, lane 3). Following SDS-PAGE of a larger quantity of peak 1a, the ~100-kDa band was excised and subjected to trypsin digestion, and N-terminal sequences of two HPLC-purified peptides were obtained. These showed the sequences Gly Arg Gly Ala Pro Gly Gly Ala Gly Ala Arg and Gly Pro Ala Gly Pro Gin Gly Pro Arg Gly, which are unique to the \( \alpha_1 \) chain of type I collagen, and not found in any other known protein, including multiple other collagens.

### Table I. Enhanced expression of \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) integrins on T cell lines vs freshly harvested, resting peripheral blood T cells from the same donor

<table>
<thead>
<tr>
<th></th>
<th>( \alpha_1 )</th>
<th>( \alpha_2 )</th>
<th>( \alpha_3 )</th>
<th>( \alpha_4 )</th>
<th>( \alpha_5 )</th>
<th>( \beta_1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting CD4(^+) PBL(^b)</td>
<td>0.6 (15.6)</td>
<td>8.8 (17.6)</td>
<td>3.4 (16.6)</td>
<td>73.3 (35.2)</td>
<td>21.1 (20.5)</td>
<td>87.9 (32.0)</td>
</tr>
<tr>
<td>CD4(^+) T cell line</td>
<td>62.1 (29.1)</td>
<td>95.8 (47.4)</td>
<td>70.4 (18.6)</td>
<td>81.4 (52.6)</td>
<td>93.5 (45.0)</td>
<td>96.6 (174.2)</td>
</tr>
<tr>
<td>Resting CD8(^+) PBL</td>
<td>8.8 (17.4)</td>
<td>28.3 (19.7)</td>
<td>16.4 (18.5)</td>
<td>97.7 (78.2)</td>
<td>60.1 (28.7)</td>
<td>96.4 (92.2)</td>
</tr>
<tr>
<td>CD8(^+) T cell line</td>
<td>89.8 (20.2)</td>
<td>62.7 (19.2)</td>
<td>88.1 (19.4)</td>
<td>99.4 (62.8)</td>
<td>78.7 (18.4)</td>
<td>97.6 (45.7)</td>
</tr>
</tbody>
</table>

\(^a\) Expression of integrin monomers on T cells was determined by two-color flow cytometry. Integrin-positive cells were expressed as a percentage of total CD4\(^+\) or CD8\(^+\) cells. Values in parenthesis represent mean fluorescence intensities of the integrin-positive cells relative to isotype control Ab.

\(^b\) The purity of freshly harvested CD4\(^+\) or CD8\(^+\) T cells and the T cell lines was >98%.
Discussion

The present work documents conclusively the potent costimulatory effects of type I collagen on both CD4\(^+\) and CD8\(^+\) human T cell lines. Under the conditions used, proliferation of normal peripheral blood T cells freshly purified from the same donors by positive and negative selection with immunomagnetic beads was unaffected by immobilized collagen. Thus, the responses of the T cell lines to collagen in the present work cannot be explained by significant contamination by fibronectin (1), which is capable of potent stimulation of the freshly purified cells. Furthermore, HPLC analysis revealed a substantially pure product, the major biologically active component of which yielded sequences specific for the \(\alpha_1\) chain of type I collagen following analysis of a trypsin digest. A synergistic effect of fibronectin in the human serum used during culture could also be excluded as the serum-free medium, AIM V, supported type I collagen-induced costimulation. In addition, positive selection of fresh, resting PBL with immunomagnetic beads did not lead to an altered state of activation sufficient to induce responses to collagen.

The responsiveness of the effector T cell populations to type I collagen was \(\beta_1\) integrin dependent, as shown by the highly potent inhibitory effects of 4B4 mAb. This effect of 4B4 mAb was not due to nonspecific toxicity or negative signaling, as the Ab had no effects on a T cell line proliferating under ECMP-free conditions. The type I collagen-induced responses of the T cell lines in the present experiments are largely due to VLA-1 and VLA-2 integrins, as shown by their up-regulation on T cell lines and the effects of specific blocking mAb. As the blocking specificities of

FIGURE 4. Concentration-related inhibition of responses of CD4\(^+\) (A) and CD8\(^+\) (B) T cell lines to anti-CD3 coimmobilized with type I collagen (1 \(\mu\)g/ml), by 4B4 mAb. Mean results of triplicate incubations in the presence of isotype control mAb (□) or 4B4 mAb (■) are shown. In C, a CD8\(^+\) T cell line was incubated with a range of concentrations of 4B4 mAb, immobilized anti-CD3, and either coimmobilized type I collagen (1 \(\mu\)g/ml, □), coimmobilized fibronectin (10 \(\mu\)g/ml, △), or IL-2 in solution at the concentrations indicated. Responses in the presence of IL-2 were unaffected by 4B4 mAb, which, in contrast, potently inhibited the responses to coimmobilized anti-CD3 and ECMP. In D, the effects of the indicated blocking mAb (each used at 10 \(\mu\)g/ml) on proliferation of a CD8\(^+\) T cell line in the presence of coimmobilized anti-CD3 and fibronectin (10 \(\mu\)g/ml) are shown. As the incubations were identical except for the presence of coimmobilized type I collagen (1 \(\mu\)g/ml) instead of fibronectin; blockade of T cell responses by 4B4 mAb was total. Columns labeled IgG1 and IgG3 show responses in the presence of isotype control Ab at 10 \(\mu\)g/ml. IgG2b isotype control had the same negative effect (data not shown). Abbreviations for ECMP are as in Fig. 1.

FIGURE 5. HPLC purification, SDS-PAGE, and T cell costimulatory effects of components of the type I collagen preparation. A, Reversed phase HPLC of the type I collagen preparation on a 100-mm C8 Brownlee column (peaks 1 and 2), followed by repurification of these peaks on a 250-mm C18 Phenomenex column (peaks 1a and 2a), as described under Materials and Methods. The peaks eluted at ∼35% acetonitrile. B, SDS-PAGE of the unpurified collagen preparation (lane 1) and of protein from peaks 1a (lane 2) and 2a (lane 3). The migration of standards (kDa) is shown to the left of the panel. C, Responses of a CD8\(^+\) T cell line to anti-CD3 coimmobilized with material from peak 1a (□) and peak 2a (△). Similar results were obtained in a second, independent experiment. Each point shows the mean results of triplicate 3-day assays.
available VLA-3 mAb were uncertain, no sound evidence for involvement of this integrin was found.

The CD4+ effector T cell costimulatory properties of type I collagen were supported by the findings in a more physiological in vitro system in which immobilized anti-CD3 was replaced by a collagen were supported by the findings in a more physiological in vitro system incorporating immobilized anti-CD3. This implies cell recruitment of proliferative responses of resting CD4+ T cells in the presence of immobilized collagen and the threshold concentration of Ag (Fig. 3A). Interestingly, the μg/ml potency of fibronectin in costimulating the T cell line was similar to that of type I collagen in this system, unlike the findings in the model system incorporating immobilized anti-CD3. However, the inability of type I collagen to costimulate freshly harvested, resting CD4+ PBL in the presence of immobilized autologous PBMC and the threshold concentration of tetanus toxoid directly reflects the results obtained in the assays with coimmobilized anti-CD3. The findings highlight the potential importance in T cell activation of costimulatory molecules of the skin, in which collagen type I is a major component. The results imply that therapeutic interventions targeted at interactions between T cells and ECM may be of interest, although it is currently not known whether RGD or other collagen recognition sequences (34) are involved.

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References