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Short paper

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Purified lymphocyte function-associated antigen-3 and T11 target structure are active in CD2-mediated T cell stimulation*

In this study we have used cells expressing LFA-3 or T11TS, the human and sheep forms of the ligand of CD2, as well as the purified LFA-3 and T11TS molecules themselves to study their effects on T cell activation via the CD2-mediated "alternative pathway". Sheep red blood cells, which bind to CD2 via T11TS in E-rosette formation, and human autologous monocytes, which express the LFA-3 molecule, both induce proliferation of resting T cells in the presence of *per se* submitogenic concentrations of anti-$T11_2$ plus anti-$T11_3$ monoclonal antibodies (mAb). This effect is blocked by mAb to LFA-3, T11TS and CD2 known to inhibit CD2-ligand interaction. In addition, purified LFA-3 and T11TS, when added at ng amounts to cultures containing submitogenic concentrations of anti-$T11_{2+3}$ mAb, are also strongly mitogenic for resting human T cells. Thus, both LFA-3 and T11TS are potent co-stimulators of the alternative pathway of T cell activation but by themselves do not provide a mitogenic signal. This finding is discussed with regard to a physiological role of CD2-LFA-3 interaction in T cell activation.

1 Introduction

CD2, a T cell surface glycoprotein traditionally known as the erythrocyte (E) receptor, is a cell interaction molecule [1, 2] through which an "alternative pathway" of T cell activation can be initiated [3–6]. A natural ligand of CD2 has been described in two species: T11TS is a 42-kDa sheep cell surface glycoprotein [1] with a broad tissue distribution including most blood cells [7, 8] that binds to human and sheep T cells in E-rosetting with sheep red blood cells (SRBC). The human lymphocyte function-associated antigen (LFA-3), originally defined by a monoclonal antibody (mAb) that inhibits T cell activation and T cell-mediated cytotoxicity [9], is a 60–70-kDa glycoprotein also expressed on many cell types that was recently found to bind to CD2 in T lymphocyte conjugate formation with target cells and in autologous E-rosetting [10–13]. The notion that T11TS and LFA-3 are homologous molecules in man and sheep is further supported by their very similar affinity in binding to CD2 [14] and by their serological cross-reactivity as detected by a heterologous antiserum [6, 15].

Studies using isolated CD2 and LFA-3 have suggested that the interaction between these two molecules may provide a sufficient explanation for their function in intercellular adhesion [11, 12]. On the other hand, little is known about the possible role of this interaction in T cell activation via CD2.

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Abbreviations: **E:** Erythrocyte **LFA:** Lymphocyte function-associated antigen **mAb:** Monoclonal antibody(ies) **SRBC:** Sheep red blood cells **T11TS:** T11 target structure

Initiation of T cell proliferation through the "alternative pathway" requires, in all systems studied so far, the presence of two stimuli. These can either be a pair of two antibodies to distinct epitopes on the CD2 molecule [3–5, 16], or a single anti-CD2 mAb plus phorbol myristate acetate [5]. More recently, it was shown that binding of SRBC to CD2 via T11TS also synergizes with an individual anti-CD2 mAb or with a pair of anti-CD2 mAb present at a submitogenic concentration in inducing polyclonal T cell activation [6]. This finding suggested that the interaction of CD2 with its complementary cell surface ligand may be at least one step in CD2-mediated T cell activation under physiological conditions. In the present experiments we have extended these studies to the interaction of CD2 with its human ligand, LFA-3, and investigated whether isolated T11TS and LFA-3 are also capable of synergizing with anti-CD2 mAb in polyclonal T cell activation.

2 Materials and methods

2.1 Antibodies

mAb TS2/9 [9] to LFA-3 and L180/1 [1] to T11TS were purified IgG. mAb 2PT3H9 to T11.1, 1OLD24CI to T11.2 and 1mono2A6 to T11.3 were used as ascites fluids [3, 6].

2.2 Preparation of cells

Peripheral blood mononuclear cells (PBMC) were prepared from the blood of healthy donors by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). As given in the legends to figures and tables, they were enriched for T cells either by selecting the small resting lymphocytes banding on a Percoll (Pharmacia) gradient between 50% and 60% Percoll in balanced salt solution, or by passage over a nylon wool column [17] in the presence of 5% fetal calf serum (FCS). Autologous monocytes were prepared by adhesion to glass petri dishes.

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2.3 Purification of LFA-3 and T11TS

LFA-3 was purified from Triton X-100 lysates of human erythrocytes as given in [11] by immunoaffinity chromatography on TS2/9-Sepharose. The column was washed with pH 11 buffer and LFA-3 was eluted with pH 3 buffer with 1% octyl-β-D-glucopyranoside (OG) and fractions were collected into tubes containing 0.1 vol. of 1 M Tris-HCl, pH 8.6, 1% OG. OG was removed by three cycles of ultrafiltration using a centricon-30 apparatus (Amicon, Lexington, MA) adding 2 ml of detergent-free phosphate-buffered saline (PBSP) per cycle such that the final OG concentration was $<0.01\%$. LFA-3 prepared in this manner had an apparent size of 700 kDa by gel filtration, consistent with a protein micelle containing 10 LFA-3 molecules (unpublished). T11TS was purified from Nonidet-P40 (NP40)-lysed SRBC by adsorption to L180/1 coupled to glutaraldehyde-activated glass beads (Boehringer Mannheim GmbH, Mannheim, FRG) [8]. After extensive washing in PBS containing 0.5% NP40 and 0.5 M KCl, the bound material was eluted with a 0.1 M glycine-HCl buffer, pH 3, containing 0.2% NP40. The eluate was neutralized by collecting 3.5 ml fractions into tubes containing 100 µl of 1 M Tris, pH 11.0. Fractions 2–8 wer pooled, dialyzed against $0.1 \times$ PBS + 0.02% NP40 and lyophilized. The material was reconstituted with 2 ml H_2O and dialyzed against PBS without detergent.

2.4 T cell stimulation assay

T cells were cultured in round-bottom microcultures in a final volume of 0.2 ml RPMI supplemented as given in [6]. For T cell stimulation using purified LFA-3 and T11TS, 20% FCS was used instead of 10% human serum in order to counteract residual detergent. Phytohemagglutinin (PHA; Sigma, St. Louis, MO) was used at 5 µg/ml. Cells were pulsed with the amounts and for the times given in the legends to figures and tables, and were harvested using an automated cell harvester. The incorporated radioactivity was determined by liquid scintillation counting. Standard deviations between triplicate cultures were below 15%, except at very low values.

3 Results

3.1 A costimulator assay for the effect of CD2-ligand interaction on T cell activation

When resting peripheral T lymphocytes are incubated with the CD2-specific mAb anti-$T11_{2+3}$, a marked and dose-dependent proliferative response is observed [3]. As shown in Fig. 1, the stimulatory capacity of this mAb combination is enhanced by an order of magnitude in the presence of SRBC. This strong costimulatory effect of SRBC is even observed at very low antibody concentrations at which the antibodies are not stimulatory by themselves. mAb L180/1 to T11TS, the ligand of CD2 expressed on SRBC (and on other sheep cells) [1, 8], completely abolishes this effect, suggesting that binding of T11TS to CD2 is costimulatory with anti-$T11_{2+3}$ in inducing T cell proliferation [6]. This costimulator assay thus detects a functional effect of the interaction between CD2 with a naturally occurring ligand, T11TS. In the following set of experiments, we have used this assay to investigate whether similar effects are mediated by isolated T11TS and by LFA-3, the human ligand of CD2.

3.2 Purified T11TS and LFA-3 are costimulators of the alternative pathway of T cell activation

In the experiments shown in Figs. 2 and 3 we have used purified T11TS or LFA-3 glycoproteins as a costimulus in combination with submitogenic concentrations of anti-$T11_{2+3}$ mAb. By themselves, neither these antibodies nor the purified molecules induced any proliferative response. In contrast, the combination of either T11TS or LFA-3 with the anti-CD2 mAb resulted in a marked T cell proliferation. As expected,

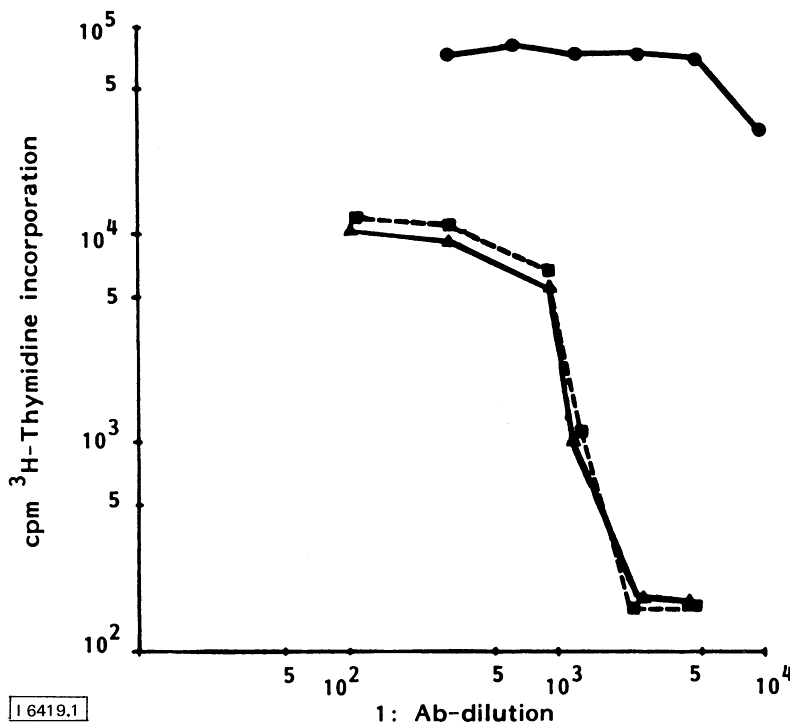

Figure 1. Costimulation of T cell proliferation by anti-CD2 mAb and SRBC. Five $\times 10^4$ Percoll gradient-purified small human lymphocytes were incubated with or without 10^6 SRBC in a total volume of 200 µl. Equal amounts of anti-$T11_2$ and anti-$T11_3$ ascites fluid diluted as given on the abscissa were present throughout the culture period. Where indicated, cultures also received 10 µg/ml anti-T11TS mAb L180/1. [^{3}H]thymidine incorporation was determined following a 16-h pulse with 1 µCi [^{3}H]thymidine at the end of the 3-day incubation period. (▲) Only anti-$T11_{2+3}$ added; (●) SRBC plus anti-$T11_{2+3}$; (■) SRBC plus anti-$T11_{2+3}$ plus anti-T11TS mAb.

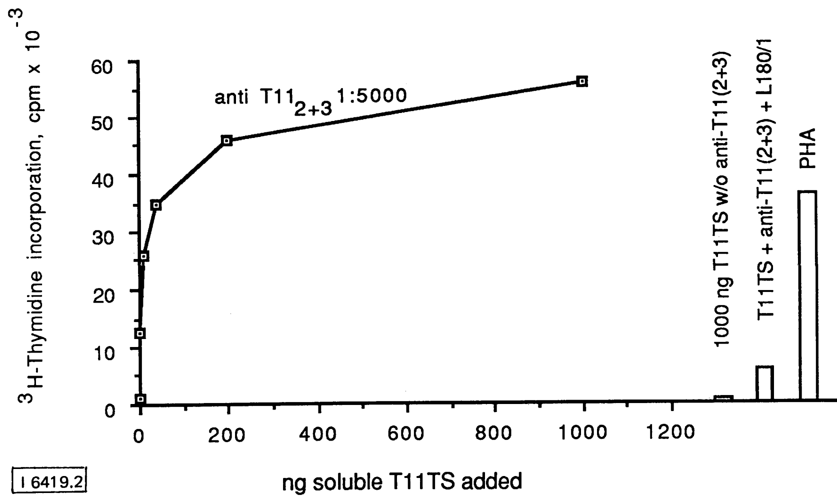

Figure 2. T cell activation by purified T11TS plus anti-CD2 mAb. 10^5 nylon wool-enriched peripheral blood T lymphocytes were cultured for 3 days in the presence of the reagents indicated. Cells were pulsed the last 16 h of culture with 0.5 μCi [^{3}H]thymidine. Anti-$T11_{2+3}$: ascites fluid at 1 : 5000 final dilution; PHA: 5 μg/ml; L180/1: 10 μg/ml.

mAb L180/1 to T11TS and TS2/9 to LFA-3 abrogated the costimulatory activity of T11TS and LFA-3, respectively, indicating that the observed effect could indeed be attributed to the presence of T11TS or LFA-3 in the costimulator assay.

3.3 Autologous monocytes act as costimulators via the "alternative pathway"

In view of the above results using isolated LFA-3 it was of interest to investigate the effects of LFA-3 in its naturally occurring form, *i.e.* as expressed on cell surfaces. Monocytes were chosen for these experiments because they represent a physiological partner for resting T lymphocytes in cell-cell interactions.

Table 1 shows the results of a representative experiment in which the capacity of autologous monocytes was studied with regard to inducing T cell proliferation in the presence of submitogenic amounts of anti-$T11_{2+3}$. While not as potent as SRBC, autologous monocytes did induce a strong proliferative response in the costimulator assay. The activity of both SRBC and monocytes in this assay was inhibited by a mAb to the $T11_1$ determinant known to block the binding of both T11TS and LFA-3 to the CD2 molecule. In addition, mAb to T11TS and to LFA-3 blocked the activity of SRBC and of human monocytes, respectively, in a species-specific fashion.

4 Discussion

The present experiments demonstrate that the binding of CD2 to either the human or sheep form of its naturally occurring ligand provides a strong proliferative signal to resting peripheral T cells in the presence of a submitogenic concentration of anti-$T11_{2+3}$ mAb. Thus, the pronounced costimulatory activity of SRBC (which are known to bind to CD2 via T11TS) and of human monocytes (which express LFA-3, the human ligand of CD2) in this assay was blocked by antibodies that interfere with CD2-ligand interaction, *i.e.* anti-$T11_1$, anti-T11TS and anti-LFA-3 mAb.

The functional activity of T11TS and LFA-3 in T cell activation via the "alternative pathway" was demonstrated directly by the stimulatory capacity of these glycoproteins when added in a purified form to the costimulator assay. We do not know at present whether these purified membrane glycoproteins exert their stimulatory function as soluble molecules or only when inserted into cell membranes. Previous experiments, in which purified LFA-3 has been shown to incorporate into E

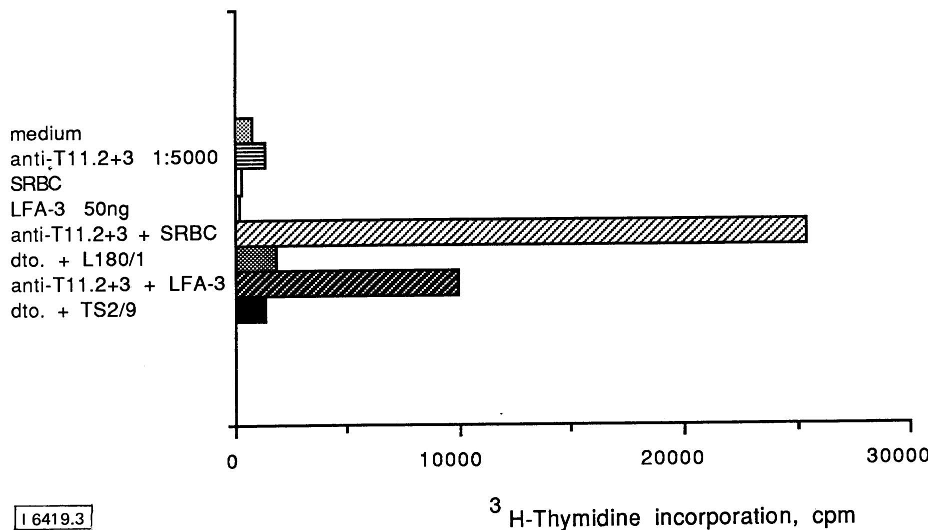

Figure 3. T cell activation by purified LFA-3 plus anti-CD2 mAb. Culture conditions were as in Fig. 2. Where indicated, 10^6 SRBC were added per well. TS2/9 (anti-LFA-3) was added at 10 μg/ml.

Table 1. mAb to T11TS and LFA-3 block the costimulatory effect of SRBC and autologous monocytes in alternative pathway activation[a)]

	Medium			Anti-$T11_{2+3}$ 1:5000		
	Medium	SRBC[b)]	Monocytes[c)]	Medium	SRBC[b)]	Monocytes[c)]
Medium	121	137	107	126	72921	8577
Anti-$T11_1$ mAb	229	115	33	86	134	190
Anti-T11TS mAb	154	86	106	111	136	12164
Anti-LFA-3 mAb	ND[d)]	121	110	ND	64554	1799

a) 5×10^4 Percoll-purified small human PBMC were cultured for 3 days in the presence of the reagents given, and pulsed with 1 μCi for the last 16 h of culture.
b) SRBC: 10^6/well.
c) Monocytes: 5×10^3/well.
d) ND = Not determined.

membranes [18], suggest that the purified ligand molecules may be incorporated into the membranes of the cells present in culture. The mode of action of purified LFA-3 and T11TS in the costimulator assay would then most likely be analogous to that of the LFA-3 naturally present on monocytes.

While the present results demonstrate a functional effect of the E receptor's natural ligand in T cell activation via the alternative pathway, it is not clear why this effect has so far only been demonstrated in the presence of anti-CD2 mAb. *In vivo*, an involvement of the CD2-LFA-3 system in T cell activation beyond its function in intercellular adhesion would thus have to involve either different types of interacting cells or additional signals that were absent from the present experimental system. The following possibilities come to mind. (a) T cells may respond directly to the engagement of CD2 with LFA-3 only at a certain stage of differentiation, *e.g.* during thymic ontogeny. No conclusive results are available at present to support this concept. (b) In contrast to SRBC or monocytes as employed in the present study, other types of "accessory cells" may, through their LFA-3 molecules, be able to trigger T cells via CD2 without additional anti-CD2 mAb. Such a mechanism may be operative in some cases of "auto-MLR" without an overt antigen (anti-self) specificity. (c) The binding of CD2 to its ligand may deliver a signal that only functions cooperatively with a signal mediated through the antigen-receptor complex. For instance, antigen-presenting cells expressing high levels of LFA-3 may facilitate the activation of low affinity T cell clones by providing supportive stimulation through CD2. The recent findings of Yang et al. [19] may support such a mechanism. (d) Finally, the possibility must be considered that LFA-3/T11TS is one, but not the only ligand of CD2. Thus, interaction of CD2 with LFA-3 could induce a conformational change in the molecule that exposes the binding site for a second ligand, mimicked in our experiments by anti-$T11_3$ [6] or by anti-$T11_{2+3}$.

The CD2-LFA-3/T11TS system is uniquely amenable to analysis because the receptor and (at least one of) the ligand molecules are defined by mAb. Moreover, the availability of the purified receptor [12] and ligand ([11], and this study) in a biologically active form now provides potent tools to address some of the questions raised above.

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