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Anti-leucocyte function-associated antigen-1 antibodies inhibit T-cell activation following low-avidity and adhesion-independent interactions

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SUMMARY

Anti-leucocyte function-associated antigen-1 (LFA-1) antibodies can provide either stimulatory or inhibitory signals to T cells, depending on the epitope they recognize, type and stage of activation of the T cells, and nature of the activation stimulus. Because of the low affinity of interaction between the T-cell receptor (TcR) and the antigen/major histocompatibility complex (MHC), it was proposed that the LFA-1 molecule strengthens the adhesion between the interacting cells, thus contributing in an additive manner to TcR-specific interactions. To check if high-avidity, TcR-specific interactions still require the accessory function of the adhesion molecule, we studied the effect of anti-LFA-1 antibodies on T-cell triggering mediated through chimeric receptors composed of an Fv of an antibody and a constant region of the TcR. Such chimeric TcR (cTcR) confer on T cells antibody-type specificity and affinity. We made use of transfected T-cell hybridomas expressing various amounts of either one cTcR chain (composed of V_H linked to $C\beta$) or double-chain cTcR ($V_HC\beta + V_LC\alpha$). When such transfectants were stimulated with hapten-modified cells, anti-LFA-1 antibodies inhibited activation predominantly mediated through cTcR composed of a single chimeric chain and did not inhibit stimulation of the double-chain transfectants. Moreover, these anti-LFA-1 antibodies blocked antigen-specific T-cell activation regardless of whether the stimulus was adhesion dependent or not, such as in the case of stimulation by immobilized hapten–protein conjugates. These studies show that the 'off-signal' provided by anti-LFA-1 antibodies is adhesion independent and affects mainly low-avidity TcR–antigen interactions.

INTRODUCTION

The leucocyte function-associated antigen-1 (LFA-1) molecule, one of the members of the leucocyte adhesion protein subfamily, plays a key role in cell adhesion among lymphocytes.[1] Recently, it was also found to be involved in signal transduction in T cells and natural killer cells.[2] A close relationship has been observed between the LFA-1 molecule and the T-cell receptor (TcR)/CD3 complex:[3] anti-LFA-1 monoclonal antibodies (mAb) were found to have a regulatory effect in TcR-mediated T-cell proliferation[4,5] both in human peripheral blood lymphocytes (PBL) and T-cell clones. Moreover, it was shown that cross-linking of the TcR/CD3 complex can modulate LFA-1-mediated adhesion,[6] virtually by increasing LFA-1 affinity for its intracellular adhesion (ICAM-1) molecule-1 ligand. Because the affinity of TcR-mediated interactions is considered to be very low, it is generally accepted that the major contribution of LFA-1 is in strengthening the adhesion between the T cell and the antigen-presenting cell (APC). It was therefore of interest to assess the role of LFA-1 under conditions of cellular interactions with high affinity.

We have recently developed a system in which chimeric TcR (cTcR) genes composed of immunoglobulin variable gene segments linked to TcR constant gene segments could be functionally expressed in T-cell hybridomas.[7,8] Using cTcR genes containing variable domains of the anti-TNP Sp6 monoclonal antibody (mAb),[9] either in association (V_H + V_L) or alone (V_H), we could obtain transfected hybridomas that expressed various levels of cTcR which bound TNP with different avidities. This system has provided us with a unique way to study the relationship between LFA-1 and the avidity of TcR-mediated interactions in the modulation of T-cell activation. Understanding the inhibitory mechanism by which anti-LFA-1 antibodies block T-cell responses has become of special interest in view of recent application of anti-LFA-1 mAb for the

Abbreviations: APC, antigen-presenting cells; cTcR, chimeric TcR; C, constant region; ICAM-1, intercellular adhesion molecule-1; IL-2, interleukin-2; LFA-1, leucocyte function-associated antigen-1; MHC, major histocompatibility complex; mAb, monoclonal antibodies; TcR, T-cell receptor; TNP, 2,4,6-trinitrophenyl; Fv, variable region of antibody composed of both heavy (V_H) and light (V_L) chains.

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Table I. Cells and level of expression of transfected cTcR genes

Transfectant code	Chimeric chain expressed* $V_HC_{(x)}$	$V_LC_{(x)}$	Recipient cell
GTA-E20	+++ (β)*	+++ (α)	MD45
GTA-H4	+++ (β)	+ (α)	MD45
GTA-D	+++ (β)	−	MD45
AD-4	+++ (β)	+++ (α)	MD27J
GTA-G2	+++ (α)	+++ (β)	MD45
GTA-C	+++ (α)	−	MD45

The anti-TNP-specific cTcR genes were introduced by electroporation into either MD45, an H-2D^b allospecific CTL hybridoma, or its α-chain-deficient mutant MD27J. Most MD45 transfectants (excluding GTA-G2) retained their original anti-EL4 specificity and were bispecific. MD27J transfectants displayed only the antibody-type anti-TNP specificity. Transgene expression was assessed at the RNA and protein level.[7]

* Letters in parentheses specify the α or β origin of the constant region in the chimeric chain.

specific immunosuppression of recipients of allogeneic bone marrow grafts.[10]

To this end, we analysed the effects of anti-LFA-1 mAb on the activation of transfectants expressing relatively high- or low-affinity cTcR following antigen-specific triggering with either hapten-modified stimulator cells or cell-free, adhesion-independent stimuli coming from immobilized hapten–protein conjugates. The results obtained clearly indicate that anti-LFA-1 antibodies primarily inhibit T-cell activation via the low-avidity interactions and in a manner which is independent of intercellular adhesion.

MATERIALS AND METHODS

Chimeric TcR-expressing hybridomas

All the effector cells used in this study were derived from the MD45 cytotoxic hybridoma[11] or the MD27J, an α-chain negative variant derived from MD45.[8] These cells were rendered 2,4,6-trintrophenyl (TNP)- specific following their transfection with cTcR genes composed of the V gene segments of the anti-TNP Sp6 mAb[12] fused to the C gene segments of the TcR chains. The construction of the cTcR genes, their transfection and expression in the hybridomas were detailed in our previous publications.[7,8] The transfectants used in this study are described in Table 1. Cells were maintained in selective medium composed of Dulbecco's minimal essential medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% foetal calf serum (FCS), 1% HEPES buffer (Biolab, Jerusalem, Israel), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM glutamine, 1 mM non-essential amino acids (Gibco), 50 U/ml penicillin, 50 μg/ml streptomycin and containing either 2 mg/ml of G418 (Geneticin, Gibco) or a combination of G418 and 1 μg/ml mycophenolic acid in addition to 15 μg/ml hypoxanthine and 200 μg/ml xanthine (Sigma, St Louis, MO).

Flow cytometry analysis

2×10^6 cells were incubated in a final volume of 200 μl of phosphate-buffered saline (PBS)/0·1% bovine serum albumin (BSA), for 20 min on ice, with (1) H57 597, hamster anti-mouse TcR β-chain,[13] (2) M17/4, rat anti-mouse LFA-1,[14] or (3) 20.5, mouse anti-mouse Sp6 idiotype[15] mAb. The cells were then washed twice and incubated under the same conditions with the appropriate secondary reagents: fluorescein isothiocyanate (FITC)–goat anti-hamster or FITC–goat anti-rat or FITC–goat anti-mouse antibodies (BioMakor, Rehovot, Israel). Control samples were incubated with secondary reagents alone. After three washes the samples were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Five thousand viable cells were analysed in each sample.

IL-2 release assay and inhibition studies

For stimulation with TNP-modified targets: 10,000 rads irradiated A.20 (a BALB/c B-lymphoma cells) were mixed for 10 min at 37° in a final concentration of 10 mM 2,4,6-trinitrobenzene–sulphonic acid (TNBS), at pH 7·4, followed by extensive washing as described previously,[16] and co-cultured for 24 hr at various stimulator:effector cell ratios with 5×10^4 transfectants in a final volume of 200 μl of the culture medium described above (without the selective drugs). All tests were performed in triplicate cultures using flat-bottom 96-well microtitre plates (Nunc, Roskilde, Denmark). For stimulation with immobilized antigen, flat-bottom microtitre plates were coated for 2 hr at 37° with 200 μl of TNP–BSA at indicated concentrations in PBS, washed three times with PBS and then blocked for another 2 hr at 37° with PBS/1% BSA and washed again intensively with PBS. In each case, 5×10^4 transfectants/well were tested in a final volume of 200 μl, for a 24-hr stimulation.

In the anti-LFA-1 inhibition studies, serial dilutions of purified M17/4 and M17/5.2 rat mAb[14] were prepared in the assay wells just before addition of the cells. Purified IgG of the rat anti-mouse FcγRIII mAb 2.4.G2[17] was used as a control.

The content of IL-2 in supernatant following stimulation was determined using the interleukin-2 (IL-2)-dependent CTL-L cell line and the methyltetrazolium assay, as described previously.[18] Results are expressed in terms of optical density difference between the absorbance measured at 690 and 540 nm. Maximum stimulation for all clones resulted in an $OD_{690-540}$ between 0·45 and 0·6. In parallel with the tested supernatant, serial dilutions of recombinant human IL-2 (Cetus Corp., Emeryville, CA) were used as controls. The OD values reported were obtained on a linear portion of the titration curves where optical density was proportional to supernatant concentration. All results are expressed as the mean of triplicates. SEM never exceeded 10% of the mean value.

Determination of antigen-binding properties of solubilized cTcR

To determine the relative affinity of the different cTcR, we measured the ability of TNP-ε-amino caproic acid to inhibit the binding of TNP–BSA of solubilized cTcR. Washed pellets containing 10^8 cells were lysed in 1% digitonin, 0·12% Triton X-100 in 10 mM Tris–HCl–saline buffer pH 7·4 containing 10 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride (Sigma), 10 μg/ml aprotonin and 10 μg/ml leupeptin hydrogen sulphate (Boehringer Mannheim, Indianapolis, IN). After 20 min at 0° and centrifugation at 12,000 *g* for 15 min, 100 ml aliquots of serial dilutions (in the lysis buffer) of lysates from transfectants and control cells were added to microtitre plates precoated with 10 μg/ml of TNP–BSA. Following 2 hr incubation at 4° and washings with 0·05% Tween-20–PBS, hamster anti-α or β TcR

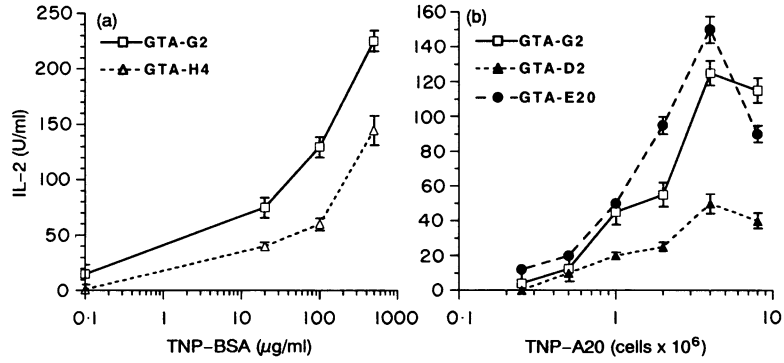

Figure 1. Stimulation of transfectants expressing different combinations of cTcR chains by either immobilized TNP–BSA (a) or TNP-modified A.20 lymphoma cells (b). The cTcR composition expressed by the transfectants is described in Table 1. One million effector transfectants were incubated with irradiated TNP–A.20 stimulator cells or wells coated with different concentrations of TNP–BSA. The amount of IL-2 produced was determined after 24 hr.

mAb[13] were added for an additional 2 hr incubation at 4°. Following washings, peroxidase-conjugated anti-hamster antibodies were added, plates were washed and 2,2′-azinodi-(3-ethylbenzthiazoline sulphonic acid) (Sigma), the peroxidase substrate was added. Degree of binding was determined by OD at 630 nm. The level of non-specific binding of the parental hybridomas did not exceed 10% of the binding of lysates obtained from the transfectants and was substracted. For inhibition studies, lysate dilutions that gave 70–80% binding were mixed with different concentrations of TNP-ε-aminocaproic acid (ICI, Cambridge Research Biochemicals, Cambridge, U.K.) and then added to the TNP–BSA-coated plates. Degree of binding was determined as described above. Per cent binding was calculated considering the binding of uninhibited lysate at 100%.

Immunoprecipitation and analysis of surface receptors

To determine the composition of the cell-surface cTcR, we followed the procedure described by Allison *et al.*[19] Briefly, viable transfectants and their parental hybridoma cells were surface iodinated with ^{125}I using lactoperoxidase and lysed with 1% Triton X-100 and protease inhibitors as described above. Lysates (from 60×10^6 cells) were incubated with 50 μl of ascites fluid of 20.5 anti-Sp6 idiotypic antibody for 16 hr at 4°. Following the addition of anti-mouse immunoglobulin and protein G–Sepharose (Pharmacia, LKB, Uppsala, Sweden) and additional incubation for 2 hr and four washes with the lysis buffer, the bound proteins were eluted and solubilized using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer without reducing agent. Analysis of the immunoprecipitated radiolabelled surface proteins was carried out by SDS–PAGE, using non-reducing 7·5% gel in the first dimension and reducing conditions for the second dimension.

RESULTS

Effects of anti-LFA-1 antibodies on stimulation of transfectants by TNP–A.20

To study the role of the LFA-1 molecule in T-cell activation, and the effect of the avidity of interaction between the T cell and its APC on the function exercises by the LFA-1 molecule, we made

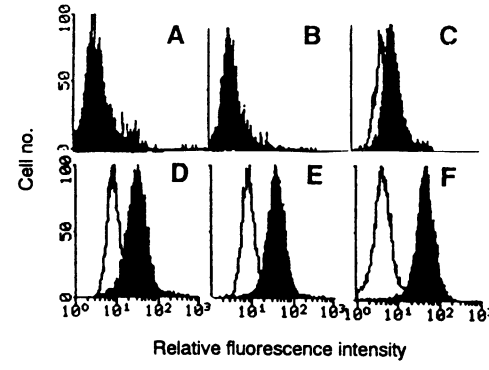

Figure 2. Cell-surface expression of the anti-Sp6, 20.5 idiotope. FACS analysis of indirect fluorescent staining. Monoclonal antibody 20.5 binds to an epitope present on the V_H of the Sp6 mAb (shaded histograms). (A) MD45 and (B) MD27J are untransfected parental cells. (C) GTA-D2, (D) GTA-H4, (E) AD-4 and (F) GTA-G2 are transfectants defined in Table 1. Solid line histograms overlaid indicate fluorescence intensity of control staining with isotype-matched mouse mAb.

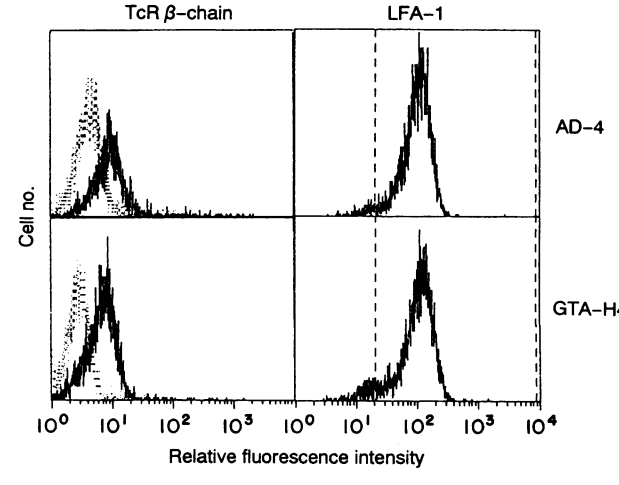

Figure 3. Immunofluorescent staining pattern of GTA-H4 and AD-4 transfectants with anti-LFA-1 (M17/4), and anti-β TcR (H57.597) mAb. The dotted FACS histograms indicate the fluorescence intensity of control staining with isotype-matched rat antibody.

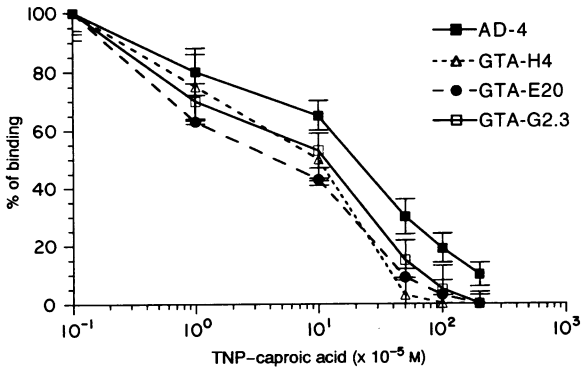

Figure 4. Inhibition of soluble cTcR binding by free hapten. Digitonin lysates from the different transfectants were added to TNP-BSA-coated wells, with and without different concentrations of TNP-ε-aminocaproic acid. Following the addition of anti-β TcR (H57.597) mAb and peroxidase anti-hamster antibodies, the per cent of binding was determined using the OD obtained in the absence of inhibitor as 100% binding.

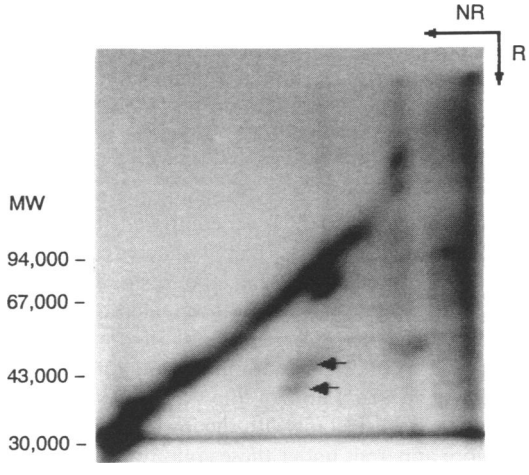

Figure 5. Antibody against the V_H of the chimeric gene co-precipitates the complementary endogenous TcR chain. Two-dimensional gel electrophoresis of ^{125}I cell-surface proteins of GTA-D2 immunoprecipitated with the anti-Sp6 idiotypic antibody 20.5. First dimension was SDS-PAGE under non-reducing (NR) conditions followed by reducing (R) conditions for the second dimension.

use of a panel of T-cell transfectants expressing cTcR which differs in its avidity towards TNP. These transfectants were generated from the MD45 CTL hybridoma by the introduction of cTcR genes encoding TcR chains in which the V region was replaced by either V_H or V_H + V_L of the anti-TNP Sp6 mAb.[9] Because the V_H can account for most of the TNP-binding capacity of Sp6 although in lower affinity than the authentic Fv (G. Gorochov and Z. Eshhar, manuscript in preparation), we chose GTA-D as a representative of a cell with low-avidity interaction with TNP. GTA-D is a transfectant expressing a single cTcR composed of $V_HC\beta$ assembled with an endogenous TcR α-chain.[7] GTA-H4 is a double transfectant which was found to express predominantly the V_H Cβ cTcR chain and very low amounts of $V_LC\alpha$.[7] Thus, GTA-H4 transfectants are expected to display an intermediate avidity for TNP. Finally, AD-4 and GTA-G2 transfectants, which express relatively high levels of both chimeric chains, were chosen for these studies because they do not display functional endogenous H-2D^b-specific TcR. These cells predominantly express chimeric receptors composed of the two complementary chimeric chains and are therefore regarded as cells with high avidity for the TNP hapten. The difference in the ability of the transfectants to respond to varying concentrations of either plastic immobilized (TNP-BSA) or cellular (TNP-A.20) antigen is illustrated in Fig. 1. This antigen-specific dose-response manifests the overall reactivity of the transfectomas. The quantitative differences shown in this experiment do not necessarily reflect qualitative differences related to the antigen-binding capacity of the chimeric receptors. A possible explanation could be that the double-chain transfectants express relatively more V_H-bearing chimeric chains than the cells transfected with a single chimeric chain encoding gene.

In order to assess the surface expression of the V_H-containing chimeric chain for each transfectant, we stained the cells with the anti-Sp6 idiotypic mAb 20.5, which recognizes an idiotope present on the V_H of the Sp6 μ-chain. As can be seen in Fig. 2, GTA-G2 cells express higher levels of the idiotope than GTA-H4 or AD-4 cells. GTA-D cells are stained to a lesser extent by the anti-idiotypic mAb. On the other hand, all transfectants used in this study expressed similar levels of TcR/CD3 and LFA-1 on their surface, as exemplified by AD-4 and GTA-H4 in Fig. 3. To verify whether affinity differences to the cTcR can account for the different pattern of responsiveness to TNP, we compared the relative affinities of solubilized cTcR isolated from the transfectants. A solubilization by mild non-ionic detergents was used in order to avoid dissociation of multimeric molecules, and the ability of TNP-caproic acid to inhibit the binding of the cell-free receptor to TNP-BSA was evaluated in a modified ELISA (Fig. 4). Indeed, we observed that TNP-bound receptor originated from the double-chain transfectants could be revealed with anti-Cβ mAb as readily as with anti-Cα mAb. As shown in Fig. 4, these double chimeric chain receptors did not differ significantly in terms of their relative affinity towards TNP; the observed IC_{50} was approximately 10^{-4} M. The degree of binding obtained from single-chain transfectants such as GTA-D2 was too low and did not allow performance of inhibition studies. The molecular nature of the receptor molecules of the single chimeric gene transfectants was determined by a two-dimensional (diagonal) analysis of surface-labelled immunoprecipitates of GTA-D2. As shown in Fig. 5, the 20.5 specifically precipitated from GTA-D2 a 85,000 MW protein which dissociated into two polypeptides of 45,000 and 40,000 MW which migrated off the diagonal after reduction. Because the replacement of Vβ with V_H does not change the molecular weight of the chimeric chain, it appears that these correspond to the TcR α-chain and the $V_HC\beta$ chimeric chain. This result suggests that the functional receptor on the surface of the single gene transfectants is heterodimer, composed of one chimeric chain bound through disulphide bond to the complementary endogenous chain.

How does anti-LFA-1 affect cellular interactions mediated by variable avidities? Figure 6 shows that activation by TNP-modified A.20 stimulator cells of the double cTcR chain expressing AD-4 and GTA-G2 transfectants is not blocked by saturating amounts of anti-LFA-1 antibodies. In contrast, anti-

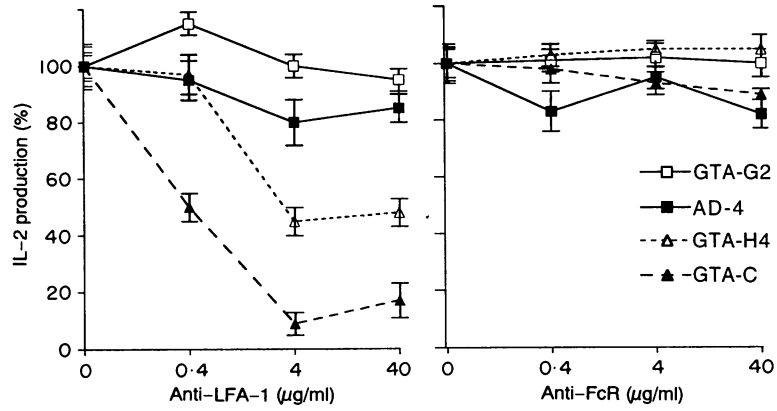

Figure 6. Differential inhibition by anti-LFA-1 mAb of cellular stimulation of transfectants displaying different avidities for TNP. Purified anti-LFA-1 (mAb M17/4) or anti-FcγR (mAb 2.4.G2, as control) were added to the transfectants just before the addition of irradiated TNP–A.20 cells at E/T of 1/5. Following 24 hr incubation the content of IL-2 in the supernatants was evaluated. The amount of IL-2 produced in the absence of antibody was taken as 100% IL-2 production. The results depicted represent one experiment carried out in triplicate. A similar pattern of inhibition was obtained with another anti-LFA-1 mAb (M17/5.2).

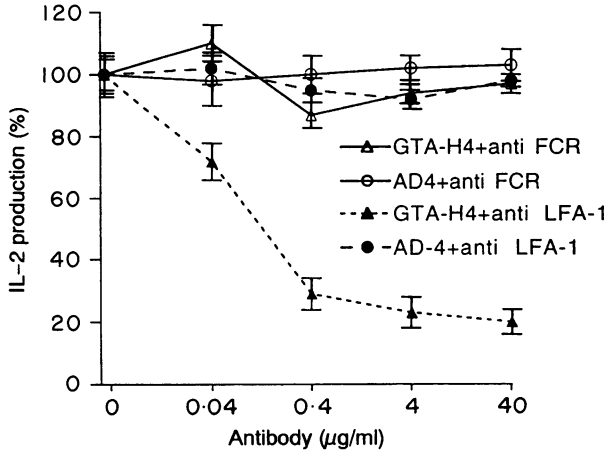

Figure 7. Effect of anti-LFA-1 antibody on stimulation of transfectants with plastic-immobilized antigen. Wells of microculture plates were coated with 25 μg/ml TNP–BSA and then purified antibodies and cells were added. Antibodies used and IL-2 production assay are as described in Fig. 6.

LFA-1 antibodies inhibited the activation by TNP–A.20 cells of transfectants expressing only, or predominantly, single cTcR chains such as GTA-C. The same pattern of behaviour was observed with additional transfectomas: double-chain cTcR transfectomas such as GTA-E,F[7] were not inhibited by anti-LFA-1 mAb, while single-chain ones like GTA-D were readily blocked (data not shown). An irrelevant mAb (rat IgG anti-mouse FcγRIII, which binds to the transfectomas and has the same IgG subclass as the two different anti-LFA-1 mAb tested) had no significant effect on the stimulation of the transfectants.

Effects of anti-LFA-1 antibodies on activation of transfectants by non-cellular antigens

LFA-1 is known to play a key role during cell–cell interaction. It was of interest to determine whether the selective inhibitory effect of anti-LFA-1 mAb is restricted to activation modes employing cells as stimulators, or if these mAb could also affect the activation of transfectants by cell-free antigen. In a previous study,[7] we have shown that cTcR-bearing transfectants can be efficiently stimulated by hapten–carrier conjugates only when immobilized on a solid support. In the experiment described in Fig. 7, we tested the ability of anti-LFA-1 antibodies to inhibit the activation of the transfectants by plastic immobilized TNP–BSA. As shown in Fig. 7, under conditions of antigen excess, the anti-LFA-1 mAb inhibited the IL-2 production by GTA-H4 but not by AD-4 transfectants.

To verify whether this reflects qualitative or quantitative differences amongst these cells, we compared their behaviour after varying levels of stimulus. To this end, we coated the wells of the culture plate with increasing amounts of TNP–BSA and studied the ability of the transfectants to undergo stimulation in the presence of anti-LFA-1 antibodies. Figure 8 shows that under suboptimal conditions of stimulation, anti-LFA-1 mAb also inhibited the activation of the AD-4 transfectant. The inhibition of the transfectant with the lower avidity, GTA-H4, becomes more apparent under these conditions.

DISCUSSION

The main result of this study is that anti-LFA-1 antibodies inhibit the activation of T cells in response to low-avidity TcR–antigen binding, even in the absence of cellular LFA-1 × ICAM-1 interactions. Although it is well established that under physiological conditions, the accessory function of LFA-1 is mediated by reinforcing the adhesion of T cells to their targets through binding with ICAM-1 and ICAM-2 on the antigen-bearing cells, the inhibitory activity of T-cell responses by anti-LFA-1 antibodies could not be entirely by interference with the adhesiveness between the interacting cells.[20] Recently it has been shown that anti-LFA-1 antibodies could inhibit the proliferative response of T-cell clones following stimulation by immobilized anti-CD3 antibodies independently of cell–cell interactions.[4,5] The results obtained in our study confirm these observations by demonstrating that stimulation of T cells expressing cTcR by immobilized antigen is also inhibited by anti-LFA-1 antibodies (Figs 7 and 8). Moreover, we show here that the inhibitory signal provided by anti-LFA-1 mAb can be overridden by TcR signalling induced by interactions of high avidity (Fig. 6).

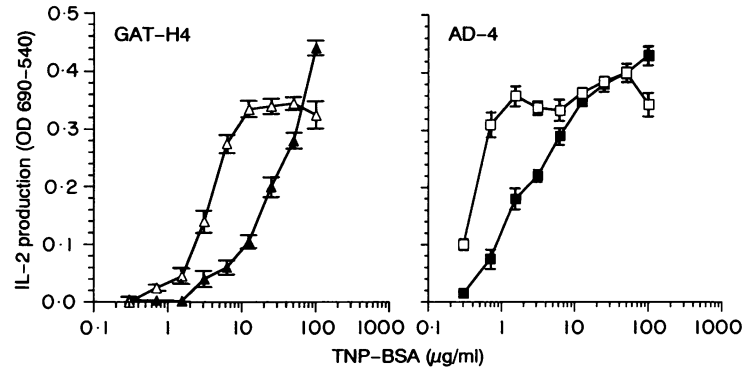

Figure 8. Anti-LFA-1 inhibition of stimulation of transfectant expressing low amounts of $V_HC\beta$ chain (GTA-H4) is more pronounced than that of high cTcR expressing cell (AD-4). Fifty per cent inhibition was evident at stimulation by 20 μg antigen for GTA-H4 and at a log lower for AD-4. Different amounts of TNP–BSA were used to coat the wells of polystyrene microculture plates. Following washing, 20 μg/ml of anti-LFA-1 (closed symbols) and 5×10^4 transfectants were added to each well. IL-2 production after 24 hr was monitored by the MTT colorimetric assay.

The experimental system we employed, utilizing a TcR with the antigen-binding site of an antibody, allowed us to obtain T cells that interact with their antigen both in cell-bound or cell-free form and in an MHC-independent manner. This same system provides us with a controlled approach to obtain T cells with a defined avidity. Thus, we made use of the fact that T-cell hybridomas expressing a cTcR composed of a single V_H-containing chain are less responsive to antigen than the double-chain (V_H+V_L) cTcR-expressing hybridomas. The fact that replacement, or omission, of the antibody light chain significantly decreases the antigen-binding affinity of antibodies,[21] would argue for an expected similar behaviour in cTcR in which the $V_HC\beta$ (or $V_HC\alpha$) chain is paired with the complementary endogenous TcR chain, like those expressed by GTA-H4 and GTA-D transfectants. Indeed, after solubilization, all the double-chain receptors had similar affinities for the hapten TNP–caproate (Fig. 4), while the receptors consisting of a single chimeric chain could hardly bind to the immobilized TNP–BSA under the same experimental conditions. The differences in activity and number of surface cTcR of the transfected cells (Figs 1 and 2) suggest that receptors composed of two complementary chimeric chains are better expressed than those consisting of one chimeric and one endogenously encoded TcR chain (Fig. 5). This probably results from preferential pairing of the complementary chimeric chains over the chimeric–endogenous chain combination. Taking all these factors together, the transfectants studied here represent three groups in their relative avidity toward TNP: the double chimeric chain expressing GTA-G2, GTA-E20 and AD-4 transfectants have higher avidity than GTA-H4 (expressing only few double cTcR chains and more mixed-chain receptors), the lowest avidity cells are the single-chain transfectants like GTA-D which express only mixed-chain receptors.

The affinity[22] of Sp6 mAb for TNP is $1{\cdot}4 \times 10^4$ M^{-1}. Such an affinity, as well as the apparent affinity of the solubilized double-chain cTcR (approximately 10^4 M^{-1}; Fig. 4), falls within the range reported for genuine TcR.[23] Because it has been generally accepted that stabilization of T cell–APC interaction through the LFA-1 × ICAM-1 may be required for T-cell activation at low TcR occupancy and with low affinity,[1] it was of interest to test whether our cTcR transgene-expressing T cells could function independently from adhesion molecules, and particularly from LFA-1. Like others, we have previously shown that anti-LFA-1 mAb completely block the cytolytic activity of the MD.45 hybridoma,[24] the cell into which the cTcR genes were introduced. In this study, we show that the same antibodies, even in excess, did not inhibit the reactivity of transfectants expressing high levels of the two chimeric chains, for example AD-4 and GTA-G2 (Fig. 6). On the other hand, partial but significant inhibition was observed with transfectants displaying only, or predominantly, mixed receptors containing the $V_HC\beta$ and the TcR α-chain.

The cTcR-expressing cells are unique in their ability to be activated by hapten bound not only to APC, but also to any solid phase. Triggering through immobilized antigen occurs without an adhesion step, as there is no other ligand for the cellular receptors (such as ICAM-1, ICAM-2 or MHC molecules) except the plastic-bound TNP. Under these conditions, anti-LFA-1 antibodies decreased the antigen-induced cell activation. Although under conditions of optimal antigenic stimulation, the anti-LFA-1 antibodies did not affect any of the transfectants expressing high levels of the two complementary chimeric chains. Under suboptimal conditions of activation, the inhibitory effect of the antibodies became more apparent. Here again, the double cTcR chain-expressing transfectants displayed higher avidity towards TNP and thereby more efficient interactions at low antigenic density than the single cTcR chain-bearing transfectants (Fig. 8). Although our experimental conditions of non-cellular, solid phase stimulation did not favour homotypic interactions between the responding T cells, we can not rule it out as a possible site for the anti-LFA-1 effect.

Our study supports the notion that there is more than one mode of action in which anti-LFA-1 antibodies can exert their inhibitory effect on T-cell function. The simplest straightforward mechanism implies that the antibodies prevent the LFA-1–ligand interaction which is essential for conjugate formation between the interacting cells. The finding that interactions of high avidity (such as those mediated by the cTcR) are not affected by the anti-LFA-1 antibodies favours the idea that the major contribution of LFA-1 to cellular interactions is merely by increasing the adherence. Yet, LFA-1 does not function only by stabilizing the T cell–APC interaction. Recent studies have demonstrated a linkage via intracellular signals between LFA-1 and the TcR.[3] It has been suggested that LFA-1 delivers a co-

stimulatory signal to T cells when the CD3 complex is engaged.[2,25] Moreover, in addition to these synergistic relationships between TcR and the ICAM-1/LFA-1 interactions, delivery of activation signals caused either by the ICAM-1/LFA-1 interaction itself,[26] or by some mitogenic LFA-1 antibodies,[4] has been implied. The same route of interaction may provide a negative signal to the T cell, and can as well be mimicked by anti-LFA-1 antibodies. The inhibition, by anti-LFA-1 mAb, of T-cell-mediated cytotoxicity of non-ICAM-1-expressing fibroblasts[27] and of anti-CD3-induced proliferation of fresh human PBL[4] or antigen-specific T-cell clones,[5] can be included in this category. The nature of the cellular proteins and intermediates which take part in the 'off-signal' or anergy transduced into the cells through binding of the anti-LFA-1 antibodies remains unclear.

The mechanism by which anti-LFA-1 antibodies interefere with the process of T-cell activation might be by disturbing the antigen-induced clustering of LFA-1 into focal areas of cell contacts. The cytoskeletal-associated molecule talin was shown to redistribute with LFA-1 to sites of antigen-specific adhesion and co-caps with LFA-1 after stimulation with PMA.[28] Interestingly, redistribution of LFA-1 and talin can be induced by very low antigen concentrations[29] which are not sufficient to lead to T-cell activation *per se*, but can favour additional co-capping of TcR and CD4 in the cell contact region.[30] We propose that interactions mediated between the high-avidity, double-chimeric receptor and the cross-linked and immobilized antigen provide a strong signal for activation which is not dependent on LFA-1, and is refractory to anti-LFA-1 antibody-mediated inhibition. When only very few receptors are engaged, we would expect co-clustering of LFA-1 molecules at the site of interaction where they could promote T-cell activation.

Together, these results strengthen the notion that LFA-1 is not only important for adhesion, contributing to enhanced conjugate formation with APC, but also acts as a signalling molecule which has intricate inter-relations with the TcR.

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