Eur J Immunol 1986 16 1289-1296

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T90/44 (9.3 antigen). A cell surface molecule with a function in human T cell activation*

T90/44 is a cell surface antigen which is present on human T cells of the helper and cytotoxic subsets and which binds the 9 3 monoclonal antibody (9 3 mAb) It is expressed in the form of 90-kDa disulfide-bonded dimers of a 44-kDa polypeptide and of free 44-kDa subunits The function of T90/44 was investigated in a series of T cell function assays 9 3 mAb was found to inhibit the activation of class II-restricted cloned T helper cells derived from leprosy patients and reactive with *M leprae* antigens The inhibition was first found at 1–10 ng/ml 9 3 mAb and regularly increased with the antibody concentration The extent of the inhibition varied among different T cell clones in proportion to the respective different levels of T90/44 expression at their cell surface The proliferative responses of peripheral blood lymphocytes (PBL) to purified protein derivative of *M tuberculosis* (PPD) and tetanus toxoid were enhanced by the 9 3 mAb resulting in up to 20–30-fold increase of [^{3}H]-thymidine incorporation After phytohemagglutinin-induced activation of PBL, the number of T90/44 molecules per cell expressed at the cell surface rose from day 0 to day 7 by a factor of about 10 High concentrations of 9 3 mAb (5–10 µg/ml) at low cell densities and in the presence of monocytes in culture media supplemented by fetal calf serum were directly mitogenic for resting lymphocytes The cytolytic effector functions of class I-restricted cytotoxic T lymphocytes (CTL) were not modulated by 9 3 mAb The mixed lymphocyte reactions of three class I-restricted CTL to their specific target cells were found not to be significantly influenced by 9 3 mAb In conclusion it is proposed that an antigen-independent T cell activation pathway can be entered at T90/44

1 Introduction

The activation of T cells to differentiate and to proliferate are essential steps in the immune response to antigen This fundamental property of the immune system correlates with the *in vitro* finding that the triggering of the antigen receptor Ti/T3-antigen complex by appropriately presented antigen or by monoclonal antibodies (mAb) is capable of eliciting proliferative T cell responses (for review see [1, 2]) The discovery that two mAb directed to epitopes of the T11 antigen in combination also can stimulate T cell growth [3] proved that additional, antigen-independent activation pathways exist More recently, it was found that yet another class of T cell surface molecules, T90/44, in response to the binding of a specific ligand, can drive T cells into proliferation T90/44 binds the 9 3 mAb [4], it is expressed at the surface of helper (T_h) and cytotoxic T cell subsets in the form of 90-kDa disulfide-bonded dimers of a 44-kDa polypeptide and of free 44-kDa subunits [5–7] The first indication that T90/44 may function in a new antigen-independent T cell activation pathway was provided by the discovery that 9 3 mAb strongly enhances proliferative cell responses to phytohemagglutinin (PHA) and allogeneic cells [5] This hypothesis was further supported by the more recent findings that 9 3 mAb also enhances the mitogenic response to 12-0-tetradecanoylphorbol 13-acetate and may act as a direct mitogen [7, 8]

To assess the functional role of T90/44 in more detail, we have investigated the effects of 9 3 mAb in cell culture assays with cloned antigen-specific helper cells as well as cytotoxic T cell lines (CTL) and resting normal T cells Our results show that depending on the type of the cells in culture, positive or negative signals can be mediated through T90/44, since the activation of helper cells by antigen was found to be inhibited by 9 3 mAb, whereas resting T cells may be activated by 9 3 mAb An important element in this functional dichotomy may be that T90/44 expression is strongly induced in activated cells The effector function of the CTL could not be influenced by 9 3 mAb, although they were found to express T90/44

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* This investigation received financial support from the Swiss National Science Foundation (Bern), Roche Research Foundation (Basel), The Foundation for Medical Research FUNGO, The Immunology of Leprosy (IMMLEP) component of the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases, the Netherlands Leprosy Relief Association (NSL) and the Nederlandse Organisatie voor zuiver-wetenschappelijk onderzoek (ZWO)

○ These investigations were carried out during a stay of W Lesslauer at the Dept of Immunohaematology and Bloodbank of the University Hospital Leiden

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Abbreviations: APC: Antigen presenting cell(s) **CML:** Cell-mediated-Lympholysis **CTL:** Cytotoxic T cells **EBV-BCL:** Epstein-Barr virus-transformed B cell lines **FACS:** Fluorescence activated cell sorter **FCS:** Inactivated fetal calf serum **FITC:** Fluorescein isothiocyanate **HA-3:** Minor histocompatibility antigen HA-3 **HS:** Heat-inactivated human pooled AB serum **H-Y:** Male specific minor histocompatibility antigen **IMDM:** Iscove's modified Dulbecco's medium **mAb:** Monoclonal antibody(ies) **MHC:** Major histocompatibility complex **PBL:** Peripheral blood mononuclear cells **PBS:** Phosphate buffered saline **PHA:** Phytohemagglutinin **PPD:** Purified protein derivative of *M tuberculosis* **SDS:** Sodium dodecyl sulfate **TT:** Tetanus toxoid

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2 Materials and methods

2.1 Cells

2.1.1 Peripheral blood lymphocytes (PBL) and Epstein-Barr virus-transformed B cell lines (EBV-BCL)

PBL were isolated from heparinized venous blood on Ficoll/Isopaque density gradients washed (×3) in Hanks' balanced salt solution (Gibco, Paisley, Scotland) and resuspended in Iscove's modified Dulbecco's medium (IMDM, Gibco) or RPMI 1640 supplemented with streptomycin 100 µg/ml, penicillin 100 U/ml, both from Flow Laboratories, Irvine Scotland, and 15% pooled human AB serum (HS) or 10% fetal calf serum (FCS) EBV-BCL were generated from 5×10^6 autologous PBL according to Steinitz et al [9] Cells were frozen in 1 ml ampoules containing 1×10^6–5×10^6 cells, 70% RPMI 1640 20% screened pooled human AB plasma and 10% dimethylsulfoxide and stored at −196 °C

2.1.2 Antigen reactivation and cloning of T lymphocytes

Cloning of T lymphocytes was performed as described elsewhere [10 11] In brief PBL of two leprosy patients (BC and R) were restimulated *in vitro* with *M leprae* in IMDM supplemented with 10% HS during 5 days at 37 °C in a fully humidified 5% CO_2-air mixture T cell blasts were obtained either by Percoll density centrifugation or by extending the cultures for another 3–10 days in the presence of 10% interleukin 2 (IL 2, Lymphocult-T, Biotest, Frankfurt, FRG) The blasts were cloned under limiting dilution conditions (0 5 cell/well) on a irradiated feeder cell layer containing autologous EBV-BCL as antigen-presenting cells (APC), and optimal concentrations of *M leprae* antigen All cultures were performed in IMDM supplemented with 10% HS in 96-well flat-bottom microtiter plates (Microtest III 3072, Becton Dickinson, Sunnyvale, CA) Growing cultures were transferred to 24-well flat-bottom plates (Falcon 3047 Becton Dickinson) and restimulated with antigen and irradiated feeder cells every two weeks Three days after each antigen feeder restimulation 20% IL 2 was added The cells were frozen 3–10 days after the final restimulation

2.1.3 CTL

The following CTL were used in the cell-mediated lympholysis (CML) assays and in the proliferative assays (a) Alloimmune HLA-A2 1 subtype-specific CTL [12] (b) HLA-A1 and B8-restricted minor histocompatibility (minor-H) antigen (*i e* minor HA-3)-specific CTL [13], and (c) HLA-A2 and/or HLA-B7-restricted H-Y-specific CTL [13]

2.2 Surface iodination, immunoprecipitation and gel electrophoresis

All cells were surface iodinated under carefully standardized conditions essentially as previously reported [5, 6] Briefly, viable cells were isolated from cultures or buffy coats on Ficoll/Isopaque density gradients and washed twice in phosphate-buffered saline (PBS) with 1 0 mg/ml glucose The cells were resuspended at either 5×10^7 or 10^8 cells/ml (cloned cells or PBL respectively) in PBS with 1 0 mg/ml glucose and lactoperoxidase (10 µl/ml of 1 mg/ml stock solution), $Na^{125}I$ (100 µCi = 3 7 MBq per 10^8 cells) and glucose oxidase (10 µl/ml of Sigma, Munich, FRG, G 6500 diluted 250×) were added The labeled cells were washed twice in PBS and lysed with 0 5% purified Triton X-100 in 50 mM Tris-HCl 150 mM NaCl, 0 02% NaN_3 (pH 7 5) at 5×10^7 cells/ml for 20 min on ice The lysate was cleared by centrifugation (400 × g, 10 min, 4 °C) and stored at −80 °C For immunoprecipitation 200 µl aliquots of the cell lysates were precleared with 10 µg purified mouse immunoglobulins (Nordic, Tilburg, The Netherlands) and precipitated with 2 5–4 3 µg of the specific mAb (30 min on ice) and 5 µl rabbit anti-mouse immunoglobulin (Z 109 Dakopatts, Copenhagen, Denmark 30 min on ice)

Fixed *Staph aureus* cells (Calbiochem, La Jolla, CA) were used as immunosorbent Reduced or nonreduced sodium dodecyl sulfate (SDS) gels were run as previously reported [5, 6] The gels were stained, dried under vacuum and exposed to Kodak XAR-5 films with Dupont Cronex Li-PLus intensifier screens at −80 °C

2.3 Antigens and antibodies

M leprae antigens were kindly provided by Dr M Abe (Nat Inst Leprosy Research, Tokyo Japan) and by Dr R C Good (Centre for Infectious Diseases, CDC, Atlanta GA) Both preparations consisted of bacilli isolated from human lepromas according to Dharmendra's procedure [14] with slight modifications The end concentrations of the first preparation in the cultures are given in µg/ml, whereas those of the second preparation are expressed as final dilution in the cultures The 9 3 mAb was originally prepared by Hansen et al [4] For the present experiments, the 9 3 mAb was bought from New England Nuclear (Boston, MA), it was purified by protein A affinity chromatography and found to be homogeneous by SDS gel electrophoresis Other monoclonal antibodies used were directed against class I major histocompatibility antigen, 9455 SA/BRL, W6/32, B9 12 1 (courtesy of Dr B Malissen), β_2-microglobulin, B11 G 6 (courtesy of Dr B Malissen), T3 antigen WT32 (RIV Bilthoven), T4, RIV6 T8, IK18, transferrin receptor, 6 8 22 1, IFA 1, 8 3 14 1, DR, B8 11 2 (courtesy Dr B Malissen), DQ, SP V L3 8 (courtesy Dr H Spits), 3A1-like determinant, 6 4 5 1 and $C3b_i$, PdV 10 2

Goat anti-mouse fluorescein isothiocyanate(FITC)-conjugated immunoglobulin was purchased from Nordic Unless otherwise specified, all antibodies were used at previously determined optimal concentrations

2.4 Cellular assays

2.4.1 Activation of cloned human T helper cells by *M leprae*

One × 10^4 TLC and 5×10^4 irradiated (40 Gy) autologous or HLA class II-matched peripheral blood lymphocytes (PBL) as APC in IMDM with 10% HS were cultured with *M leprae* antigen in optimal concentrations in 96-well flat bottom microtiter plates (Greiner, Nurtingen, FRG) Phytohemagglutinin (PHA, Wellcome Diagnostics, Beckenham, GB, 4 µg/ml) and plain IMDM were used as controls 9 3 mAb was taken up in RPMI 1640 with 20% HS and dialyzed against RPMI 1640 Various amounts of the dialyzed antibody were added to the

cultures calculated to give final concentrations ranging from 0 001–10 μg/ml antibody

The cultures were set up in duplicate or triplicate and incubated as described above for 72 h Eighteen hours before termination 1 0 μCi = 37 kBq of [methyl ^{3}H]thymidine ([^{3}H]dThd spec act 5 0 Ci/mmol Radiochemical Centre Amersham, GB) in 0 05 ml RPMI 1640 was added The samples were harvested on glass fiber filters using a semi automatic device [^{3}H]dThd incorporation was assessed by liquid scintillation counting As a control parallel cultures were set up with the anti 3A1 like antibody 6 4 5 1 in comparable dilution directly from pure ascites

2 4.2 Mixed lymphocyte culture (MLC)

MLC were set up by culturing 50000 responder and 50000 irradiated (2000 rds) stimulator cells in V well microtiter trays in 0 15 ml of RPMI 1640 supplemented with 15% pooled human AB serum and 50 μg/ml gentamycin (Flow Lab Irvine Scotland) The cells were cultured for 120 h, 18 h before harvesting, 1 μCi of [^{3}H]dThd was added to each culture Antibodies were added at the start of the MLC and left throughout the culture period

2 4.3 Lymphocyte transformation tests

Lymphocyte transformation tests were carried out as follows 50000 responder cells (PBL or fluorescence activated cell sorter (FACS) separated cells) were cultured in flat bottom microtitertrays in 0 15 ml/well of tissue culture medium (RPMI 1640, gentamycin 50 μg/ml 15% HS) containing either 5 μg/ml purified protein derivative of tuberculin (PPD Statens Serum Institute Copenhagen) 0 75 I f/ml tetanus toxoid (TT, RIV Bilthoven The Netherlands) or 5 μg/ml PHA Culture, incubation time and [^{3}H]dThd incorporation were carried out as described in Sect 2 4 2 PBL activated by PHA to be labeled and immunoprecipitated at day 0 3 5 7 were treated as above but 20% interleukin 2 (IL 2) was added to the medium after day 3

2 4 4 Cell-mediated lympholysis (CML) assay

The alloimmune and the MHC restricted CTL populations were generated according to the culture procedures described earlier [12 13] The effector cells were pre incubated for 30 min with three different concentrations of the 9 3 mAb before addition of the specific target cells The CML assay has been described in detail [13 15] The percentages of lysis were determined in relation to PHA stimulated blast cells in a 4 h ^{51}Cr assay Cytotoxicity (*i e* the amount of isotope released from ^{51}Cr labeled target cells) was determined and calculated according to the described method [13] Standard errors of the mean of triplicate determinations were less than 5% Positive and negative assignments were made on the basis of a 10% specific ^{51}Cr release value All experiments were repeated at least twice at different effector to target ratios

2.5 Indirect immunofluorescence and FACS analysis

Analysis of indirect surface immunofluorescence and cell sorting were carried out with a FACS IV (Becton Dickinson Sunnyvale CA) as reported elsewhere [16]

3 Results and discussion

3.1 Inhibition of response to antigen of cloned T_h cells

Class II restricted cloned T_h cells specific for *M leprae* antigens were derived from leprosy patients as previously reported [10 11] The activation of a number of these T cell clones by *M leprae* as a function of the 9 3 mAb in the concentration range of 0 001–10 μg/ml was measured by incorporation of [^{3}H]dThd into macromolecular nucleic acids It was found that the 9 3 mAb strongly inhibited the antigen responses in some of these clones (*e g* clones 4A4 2F9 2F10) whereas the inhibition in other clones was weak (*e g* clone 6C 7) or practically absent (clone 2G11 see Fig 1) In these latter cases inhibition was observed only at very high 9 3 mAb concentration In the strongly inhibited clones the inhibitory effect was first observed at ng/ml 9 3 mAb and regularly increased with the antibody concentration The practically constant negative

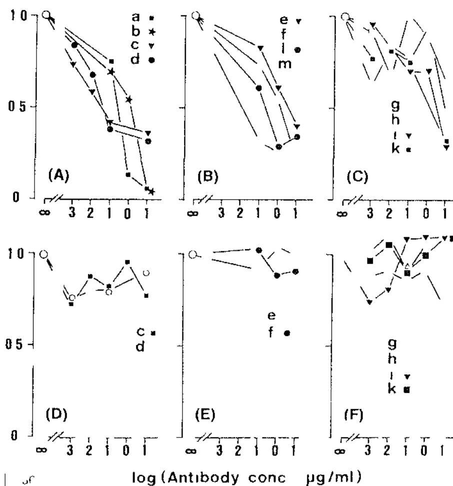

Figure 1 Inhibition of the activation by specific *M leprae* antigens of cloned human T cells by 9 3 mAb in the concentration range of 0 001–10 μg/ml antibody (A–C) Parallel cultures with the 6 4 5 1 mAb (D F) were included as control for an irrelevant antibody of the same IgG subclass as 9 3 mAb The data are expressed as relative inhibition according to

$$\left(1 - \frac{cpm_c - cpm}{cpm}\right)$$

(cpm = incorporated [H]dThd without antibody cpm n incorporated [^{3}H]dThd in the presence of antibody of concentration i) The range of the actually measured counts was 10 cpm 10 medium controls were typically < 500 cpm All clones were found to respond strongly to PHA (data not included) Each curve represents the mean of triplicate cultures of one independent experiment standard errors were ≤ ± 0 25 (A D) clone 4A4 (a – 2 μg/ml b 4 μg ml *M leprae* c = 1/120 dilution d 1/60 dilution dharmendra) (B E) clones 2F9 (e l) and 2F10 (f m) (1/120 dilution dharmendra) (C F) clones 2G11 (g h) and 6C7 (i k) (g 1 120 dilution h k 1 60 dilution dharmendra) Antigen presented by irradiated autologous PBL (a k) or by EBV BCL (l m)

slope of the dose response curve in these clones contrasts significantly with the practically antibody independent responses in parallel cultures with the 6 4 5 1 mAb which for the purpose of the present investigation may be considered as an irrelevant control antibody of the same IgG subclass as the 9 3 mAb The negative result of this latter control experiment allows to rule out that the effect of 9 3 mAb is due to trivial phenomena such as antibody dependent cytotoxicity

All curves in Fig 1 represent independent experiments which have been carried out during a period of about one year Within that period the extent to which a given clone responded to the inhibitory effect of the 9 3 mAb in the *M leprae* stimulation assay was found to be reproducible the response to the 9 3 mAb thus appears to be a specific and stable property of the individual clones at least within that period It is worth noting that all these clones in functional assays behave as class II restricted T_h cells [10 11] furthermore they all respond well to mitogenic stimulation by PHA (data not shown) The different responses in the assays presented in Fig 1 therefore are not due to differences in the general proliferative potential among the clones

3 2 9 3 mAb inhibition of *M leprae* activation is proportional to T90/44 expression of the different T_h cell clones

To discover the cause of the variability in the 9 3 mAb dependent inhibition of the activation by *M leprae* among the different cell clones the level of expression of T90/44 and of a number of reference antigens was investigated Lysates of surface iodinated cells were sequentially immunoprecipitated with a panel of mAb the immunoprecipitates were then identified in SDS gels by autoradiography as indicated in Fig 2 the bands were cut from the gels and the incorporated radioactivity was counted (Fig 3) To evaluate the data in Fig 3 LFA 1 should be considered first

In a series of preliminary experiments it had been found that the level of expression of LFA 1 when compared to that of other surface antigens is the most constant among the different cell clones This conclusion is confirmed by Fig 3 A mean of 18000 (± 6300) cpm are incorporated in the large subunit of

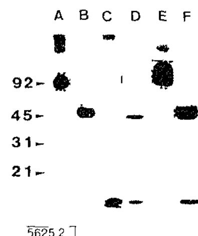

Figure 2 Immunoprecipitated T90/44 and HLA class I molecules (A) clone 4A4 T90/44 nonreduced (B) clone 4A4 T90/44 after reduction of disulfide bonds (C) clone 2G11 T90 44 nonreduced (D) clone 6C7 HLA class I (E) PBL T90/44 nonreduced (F) PBL T90/44 after reduction of disulfide bonds The precipitates in (A) (D) represent cell aliquots of 10^7 cells The precipitates in (E) (F) represent $> 10^8$ cells For the measurement of incorporated radiolabel the bands were cut out as indicated by broken lines

LFA 1 per 5×10^7 cells of the different clones The amount of radiolabel in the small subunits are similarly constant so that the ratio of radiolabel in the two subunits is 4 67 ± 0 54

The remarkable constancy of LFA 1 expression is in contrast to the HLA class I molecules where large variations among the different clones are found It is noteworthy however that HLA heavy chain and β_2 microglobulin vary to the same degree the ratio of the radiolabel incorporated in the heavy and light chains respectively is 2 59 ± 0 49 for the 6 cell clones These results support the conclusion that Fig 3 reliably represents the relative level of expression of the surface antigens when the different cell clones are compared

It appears from these data that the inhibition by 9 3 mAb of the *M leprae* activation correlates well with the level of T90/44 expression in a given clone This is most clearly seen by comparing clones 2B2 or 2F10 with clone 2G11 Clones 2B2 and 2F10 express high levels of T90/44 (5900/4300 cpm precipitated) and are strongly inhibited at ≤ 1 µg/ml 9 3 mAb clone 2G11 expresses little T90/44 (1100 cpm precipitated) and is only weakly inhibited at the highest 9 3 mAb concentration

As was to be expected from their T helper phenotype all the clones tested express similar levels of T4 antigen [10 11] It was however unexpected that clone 2G11 and to a lesser extent clone 6C7 in addition to T4 express relatively high levels of T8 antigen which does not appear to interfere with their helper function With regard to the simultaneous expression of T4 and T8 antigens in these clones it is of interest that *in further blocking studies anti T4 mAb but not anti T8 mAb* were found to inhibit the activation of clone 2G11 by *M leprae* (data not shown) The present data do not show whether the finding that the two $T8^+$ clones 2G11 and 6C7 also have a relatively low expression of T90/44 has a functional significance All the cell clones tested by the immunoprecipitations were found to express similar amounts of DRα β and DQα β

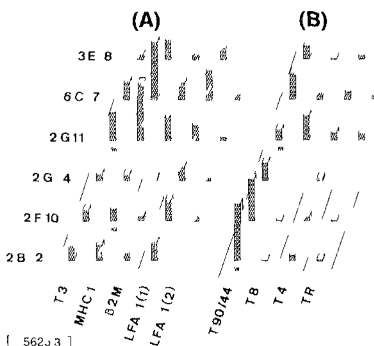

Figure 3 Incorporated radioactivity (cpm ^{125}I) in T90/44 and reference antigens immunoprecipitated from surface iodinated cell clones 2B2 2F10 2G4 2G11 6C7 3E8 (A) T3 class I antigens heavy chain (MHC I) β_2 microglobulin (β2M) LFA 1/large subunit [LFA 1(1)] and LFA 1/small subunit [LFA 1(2)] (B) T90/44 T8 T4 transferrin receptor (TR) The columns in (B) relative to those in part (A) of the figure are scaled up by a factor of 9 3 The incorporated radioactivity per 5 × 10 cells is indicated in all fields below the columns in units of cpm

(data not included in Fig 3) and of transferrin receptors (Fig 3) This finding corroborates the above conclusion from the functional assays that all the clones have a comparable proliferative potential because these surface molecules may be viewed as markers for the cellular activation state

Furthermore since the clones 2G11 (low T90/44) and 4A4 (high T90/44) were derived from the same leprosy patient BC (DR3 4/DRw52 53/DQw3/DPw1 5) whereas the other clones were derived from patient R (DR2 3/DRw52/DQw1 2/DPw5) it can be concluded that the level of T90/44 expression does not correlate with that of class II antigens

It is noteworthy that the analysis of the cell surface immuno fluorescence in the FACS IV yielded results which were con sistent with those of the immunoprecipitations in Fig 3 Therefore, the amount of radioactivity incorporated into the various cell surface antigens by the present iodination protocol also by this criterion may be considered a good approximation of their relative amounts when the different cell clones are compared A further result of the FACS analysis was that the expression of T90/44 in a given cell population is homogene ous For example the low T90/44 expression in the 2G11 cell clone is due to the equally low expression in all cells rather than to the presence of positive and negative cells because only one single peak of positive fluorescence is recorded in the FACS IV immunofluorescence profile

3 3 Effects of 9 3 mAb on cytotoxic T cell lines

Three class I restricted CTL (*i e* HLA A1 and B8 restricted anti minor H antigen specific CTL HLA A2 and B7 restricted H Y specific CTL and HLA A2 1 specific alloim mune CTL [12 13]) were used to investigate whether the 9 3 mAb is capable of interfering with cytotoxic functions It had been established by prior immunoprecipitations that these CTL express similar amounts of T90/44 as those *M leprae* activated T_h clones with high T90/44 expression (see above) when measured on a per cell basis or relative to expression of the class I antigens (data not shown) The functional effects of the 9 3 mAb and of several reference antibodies on the cellu lar cytotoxicity was investigated in a CML assay It was found that the 9 3 mAb in contrast to the reference antibodies which had various inhibitory effects was not capable of inter fering with the cytotoxic function of any of these CTL (Table 1)

In a further approach to define the regulatory mechanisms in which T90/44 plays a functional role the effects of the 9 3 mAb on the proliferative rather than the cytotoxic activities of these CTL were investigated The HLA A2 1 alloimmune and the HLA A2 restricted H Y specific CTL were stimulated in a 5 day MLR with their specific stimulator cells and co cultured with various concentrations of 9 3 mAb and anti T3 whereafter [^{3}H]dThd incorporation was measured (Table 2) The proliferation of both CTL was found not to be significantly modulated by the 9 3 mAb in contrast to the pronounced inhibitory effect of the anti T3 mAb (Table 2)

In further experiments the 9 3 mAb was tested for its ability to inhibit natural killer activity or antibody dependent cellular cytotoxicity and was found not to inhibit in those assays (data not shown)

3 4 Lymphocyte transformation tests

First indications that T90/44 has a functional role in T cell activation were provided by the finding that 9 3 mAb enhances mitogenic responses of PBL to suboptimal concen trations of PHA or to allogeneic cells whereas no direct mitogenic effect of the antibody could be detected [5] These results were confirmed and extended by Hara et al [7] but a direct mitogenic action of 9 3 mAb was documented in a more recent report [8] To investigate this point in more detail the effects of 9 3 mAb and of several reference antibodies were studied in a series of lymphocyte transformation tests carried out with PBL of 2 unrelated donors (Table 3) Strong enhancements of the proliferative responses to PPD and TT (20 30 fold) were found which support the previously reported growth enhancement phenomena [5 7] No effect of 9 3 mAb on the culture activated by PHA is detected because the PHA here is used in optimally activating concentration ([5] see Sect 2 4 3)

The above transformation tests were carried out in RPMI 1640 medium supplemented with 15% HS [5 7] However when the PBL were cultured in RPMI 1640 medium supplemented with FCS instead of HS and were exposed to 9 3 mAb without any further activating factors a strong direct mitogenic action of 9 3 mAb became apparent which nearly equalled that found with anti T3 mAb (Table 3 last column) This direct mitogenic action of 9 3 mAb is in sharp contrast with the ab sence of any activation at equivalent antibody concentrations in media supplemented by HS [5 7]

Table 1 Effects of 9 3 mAb and of reference antibodies on CML carried out with alloimmune HLA A2 1 subtype specific CTL (anti HLA A2 1) HLA A1 and B8 restricted anti minor H antigen specific CTL (anti minor HA 3) HLA A2 and B7 restricted anti H Y specific CTL and specific target cells

	CTL against HLA-A2 1		minor HA-3		H-Y	
	20 1[a)]	10 1	20 1	10 1	20 1	10 1
Medium	90%[b)]	73%	90%	75%	78%	66%
anti-T3	52% (42%)	45% (38%)	76% (15%)	60% (20%)	71% (9%)	68% (–3%)
anti-T4	88% (2%)	71% (3%)	94% (–4%)	75% (0%)	89% (–14%)	70% (–6%)
anti-T8	41% (54%)	31% (58%)	9% (90%)	8% (89%)	77% (1%)	70% (–6%)
anti-LFA-1	48% (47%)	45% (38%)	83% (8%)	56% (25%)	73% (6%)	68% (–3%)
9.3-control	70%	61%	77%	65%	60%	55%
9 3	74% (–5%)	62% (–2%)	78% (–1%)	63% (3%)	66% (–10%)	56% (–2%)

a) Effector target cell ratio
b) Percentage of target cell lysis and the inhibition of lysis (in parentheses) by the various antibodies are given as the mean of triplicate cultures in RPMI 1640/15% HS

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Table 2 Effects of 9 3 mAb and α T3 mAb on the proliferation of HLA A2 restricted anti H Y CTL (αH Y) and anti HLA A2 1 alloimmune CTL (αA2 1) in 5 day MLR with specific target cells The incorporated [^{3}H]dThd is given as the mean cpm of triplicate cultures

CTL	mAb	Antibody concentration (µg/ml)				
		Medium	0 001	0 010	0 100	1 0
anti H-Y	9 3	21010 ± 3460	24580 ± 540	21170 ± 640	20720 ± 1340	26020 ± 585
	anti T3	23045 ± 925	24965 ± 1315	8900 ± 250	3085 ± 375	2550 ± 15
anti A2 1	9 3	24560 ± 280	29510 ± 477	33060 ± 5330	38590 ± 2380	37520 ± 7135
	anti-T3	17675 ± 285	18405 ± 605	9265 ± 1550	4953 ± 32	5140 ± 10

The antibody concentration dependence and the possible role of monocytic cells in the direct T cell activation by 9 3 mAb were investigated PBL of the two donors were depleted of monocytes by sorting out cells stained with PdV 10 2 (anti monocyte) mAb on the FACS IV The cells were then cultured in medium supplemented by 10% FCS to reveal the direct mitogenic action of 9 3 mAb A strong proliferative response to 9 3 mAb again was found at 5 6 µg/ml antibody and 2 5 × 10^5 cells/ml with PBL the monocyte depleted lymphocytes did not respond to 9 3 mAb but its mitogenic action could be restored by re adding irradiated autologous PBL to the purified lymphocytes (Table 4) The cell activation triggered by 5 6 µg/ml 9 3 mAb nearly equals that in the α T3 mAb or PHA controls

This direct mitogenic action is only found at high antibody cell ratios it has practically vanished already at the fourfold higher dilution of 9 3 mAb (Table 4) where is the enhancement of growth promoting signals is detected even at ng/ml 9 3 mAb [5]

It may be concluded that the mitogenic action of 9 3 mAb depends on the presence of monocytes and is quenched by HS A likely cause of the suppression of the mitogenic action in media supplemented by HS is their relatively high content in human immunoglobulin molecules It might then be proposed that the binding of 9 3 mAb to Fc receptors of monocytes and as a consequence the possible clustering of T90/44 is functionally important This hypothesis is further supported by the recently reported finding that $F(ab)_2$ but not F_{ab} fragments of 9 3 mAb have functional effects on T cells [17] However it cannot be excluded that the quenching by HS is at least in part due to the presence of material which reacts with 9 3 mAb (*e g* shed T90/44) [5]

3.5 T90/44 expression is induced in activated T cells

Comparative immunoprecipitation studies had revealed that both the class I restricted CTL and the class II restricted *M leprae* activated T_h cell clones express significantly larger amouts of T90/44 than resting PBL It was therefore postulated that T90/44 expression is induced in activated T cells To investigate this hypothesis PBL of two unrelated donors were cultured in the presence of optimally activating concentrations of PHA and (after day 3) of IL 2 Aliquots of cells were surface iodinated at day 0 3 5 and 7 of PHA activation T90/44 together with other representative surface antigens were immunoprecipitated from cell lysates the respective bands were cut from SDS gels and the incorporated radiolabel was counted It was found that the radioactivity per cell which is incorporated into T90/44 increased regularly from day 0 to day 7 by factors of 6–10 (Fig 4) Whereas similar results were observed with DRα β and DQα β and with the transferrin receptor less pronounced increases were found with the LFA 1 or class I antigens The expression of still other surface antigens was not affected by the cell activation for example the radiolabel incorporated into the 4 3 antigen [16] remained practically constant throughout the whole activation period (Fig 4)

The surface iodination of cells and the immunoprecipitations were carried out under carefully standardized conditions they

Table 3 Lymphocyte transformation tests Effects of 9 3 mAb and of reference antibodies on proliferative responses of PBL isolated from two unrelated donors A and B elicited by PHA PPD and TT

		PHA	PPD	TT	Medium/10% FCS
Medium	A	59917 ± 1198	5292 ± 847	4857 ± 2574	273 ± 19
	B	67135 ± 24169	16170 ± 1455	9377 ± 2250	288 ± 66
Anti-T3	A	65810 ± 1316	147900 ± 7395	182933 ± 31099	63593 ± 636
	B	78490 ± 3140	127100 ± 5084	167767 ± 10066	52580 ± 2629
Anti T4	A	55913 ± 559	5988 ± 1257	1335 ± 814	298 ± 36
	B	64803 ± 1944	10067 ± 1711	6742 ± 1618	343 ± 58
Anti-T8	A	56093 ± 1122	4578 ± 1099	3522 ± 2360	138 ± 26
	B	67700 ± 1354	11700 ± 3510	4575 ± 4666	220 ± 31
Anti LFA-1	A	21840 ± 655	618 ± 272	332 ± 136	257 ± 41
	B	35003 ± 2450	2668 ± 1387	1260 ± 1575	243 ± 83
9 3 [b)]	A	61613 ± 1232	107393 ± 8591	162233 ± 22712	41330 ± 7026
	B	70383 ± 704	79780 ± 15158	58117 ± 52305	20697 ± 1656

a) Cells of two donors A and B were cultured in RPMI 1640 medium supplemented by 15% HS (PHA PPD TT) or by 10% FCS (Medium last column) Mean cpm and standard deviation (triplicate cultures) of incorporated [^{3}H]dThd

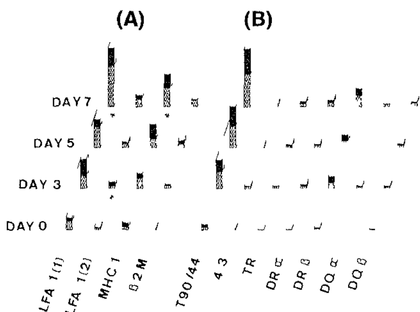
b) 5 6 µg/ml

Table 4 Mitogenic action of 9.3 mAb (5.6 and 1.4 µg/ml antibody), anti-T3 mAb and PHA in cultures of PBL (5 × 10 cells/well), lymphocytes (5 × 10⁴ cells/well) purified by sorting out monocytic cells from PBL on the FACS IV cell sorter, the same purified lymphocytes (5 × 10 cells/well) to which autologous irradiated PBL (5 × 10 cells/well) were added back, and irradiated PBL (5 × 10⁴ cells/well)

		PBL	Lymphocytes	Lymphocytes + PBL*[a)]	PBL*
Medium	A[b)]	357 ± 46[c)]	117 ± 34	262 ± 52	232 ± 16
	B	188 ± 47	102 ± 20	260 ± 159	632 ± 929
9.3[d)]	A	30087 ± 501	223 ± 120	22587 ± 2710	8095 ± 648
	B	13853 ± 693	122 ± 41	21150 ± 1480	2092 ± 209
9.3[e)]	A	1825 ± 547	123 ± 59	2815 ± 366	210 ± 57
	B	402 ± 129	93 ± 25	1098 ± 121	88 ± 20
Anti-T3	A	67350 ± 2020	355 ± 291	41183 ± 2471	15447 ± 927
	B	44183 ± 2209	212 ± 85	36390 ± 1456	6489 ± 519
PHA	A	61933 ± 1858	10950 ± 547	43433 ± 1303	20250 ± 1417
	B	58253 ± 1165	19827 ± 1586	52743 ± 2109	13740 ± 550

a) PBL* – irradiated PBL
b) Cells of two donors A and B were cultured in RPMI 1640 medium supplemented with 10 FCS
c) Mean cpm and standard deviation (triplicate cultures) of incorporated [^{3}H]dThd [illegible]
d) 5.6 µg/ml
e) 1.4 µg/ml

refer to the same activity reference date of ^{125}I. Furthermore, microscopic inspection confirmed that practically all the cells in the culture were uniformly transformed. The radioactivity incorporated into a given surface antigen thus is a good approximation for the relative number of its copies per cell when the cells of a given donor are compared throughout the activation period. It may then be concluded that the T90/44 expression increases at least in proportion with the cell surface area in the blast transformation. In view of the steeper increase of T90/44 when compared to that of class I or LFA-1, it cannot even be excluded that the cell surface density of T90/44 in the PHA blasts is higher than in resting cells.



[T 5625.4]

Figure 4. Relative expression of T90/44 and of reference antigens on PBL of two donors and in PHA activation. The antigens were immunoprecipitated from aliquots of surface iodinated PBL at day 0, 3, 5 and 7 cultured in the presence of PHA and IL 2. The lower part of the columns refers to the cpm of incorporated ^{125}I in the first donor, the total height of the columns refers to cpm incorporated ^{125}I in the second donor. (A) LFA-1/large kDa subunit [LFA 1(1)] and small subunit [LFA 1(2)], class I antigen heavy chain (MHC I), β2 microglobulin. (B) T90/44, 4.3 antigen (4.3), transferrin receptor (TR), class II antigens DRα,β and DQα,β. The columns in part (B) relative to those in part (A) of the figure were scaled up by a factor of 3. The incorporated radioactivity per 5 × 10⁷ cells of the second donor are given in all fields below the columns in units of cpm.

4 Concluding remarks

The number of the different T cell surface antigens through which the intracellular activation pathways can be addressed and which therefore can activate T cells into proliferation is small. At present the Ti/T3 complex and the T11 antigen are known to belong to this class of cell surface molecules [1, 3]. The present results together with the previous findings on the functional effects of 9.3 mAb [5, 7, 8] leave little doubt that T90/44 also can receive mitogenic information. T90/44 formally meets the essential criteria which define a receptor molecule: it is expressed at the cell surface, binds a specific ligand, and as a consequence, changes in cell function are measured. A receptor function of T90/44 therefore cannot be excluded and to search for a physiologic ligand in further studies might be justified.

There is firm evidence that T90/44 and the α/β heterodimeric Ti are distinct gene products. The subunits of T90/44 cannot be resolved into separate bands by a variety of electrophoretic methods, whereas Ti consistently is resolved into α and β subunits of different molecular mass [1, 2, 5, 6]. Furthermore, it was found that T90/44 and T3 cannot be comodulated [7, 8, 18]. T90/44 therefore is independent of the receptor structure which appears to be the complete antigen recognition site [1]. It may then be concluded that the activation through T90/44 is independent of antigen. If the time point in the T cell activation process at which T90/44 might function is evaluated, the further significant finding that 9.3 mAb directly or in combination with phorbol esters induces IL 2 and IL 2 receptor expression [7, 8] has to be considered. Any receptor function of T90/44 then would have to be placed in the period before IL 2/IL 2 receptor interaction if T cell activation is viewed as an obligatory multistep sequence of successive growth signals. However, the presently available data do not allow the conclusion that triggering of T90/44 is an obligatory step in such a sequence. The activation through T90/44 rather might represent an antigen independent bypass of the Ti/T3 complex. Models of cell activation pathways based on the latter assumption would predict that a primary activating signal received by the Ti/T3 complex may become modulated by the input of secondary subthreshold signals into accessory receptor(s). Practically all of the functional effects of 9.3 mAb reported, if they do not prove models of the latter type, at least are consistent with it.

Any model of T90/44 function has to take into account the important finding that the 9.3 mAb can either block or promote proliferation. The former is found in the inhibition of the specific response to *M. leprae* in the cloned T_h cells; the latter is clearly documented in the mitogenic effect on resting lymphocytes in the presence of monocytic cells, FCS and high antibody concentrations or in the enhanced responses to PPD, TT and PHA (Table 3; [5, 7, 8]). This apparent dichotomy of functional effects is consistent with various models. It may be envisaged that all growth-regulating signals either at the cell surface or intracellularly ultimately converge on a common biochemical pathway; if, furthermore, regulated feedback loops play a role along this pathway, it becomes obvious that depending on the status of the system the same receptor molecule may elicit positive or negative responses. The high level of expression of T90/44 in activated as compared to resting cells is likely to be one of the key factors which determine whether a positive or a negative signal is generated at T90/44.

The authors thank Dr. R. de Vries for stimulating discussions. The excellent technical assistance of Ms. Anita Hofer and Ms. Diènne Elferink is an essential part of this work. The generous gifts of monoclonal antibodies by Dr. B. Malissen (Marseille) and Dr. H. Spits (Amsterdam) are gratefully acknowledged.

Received June 20, 1986.

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