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Structural analysis of high- versus low-affinity interleukin-2 receptors by means of selective expression of distinct receptor classes

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Activated T cells express at least two distinct affinity classes of interleukin-2 (IL-2) receptors. The number of low-affinity receptors per cell is normally 10-30 times greater than that of the high-affinity receptors, and the difference in the dissociation constant between the two classes of receptors is in the order of 1000-fold. In this report normal human T cells are used in a cellular system in which the number of low-affinity receptors can be manipulated. It is demonstrated that a cell population could be achieved with such low levels of low-affinity IL-2 receptors that almost half of the surface pool of anti-IL-2 receptor antibody (anti-Tac) binding sites represented high-affinity receptors. By using this cellular system it was possible to show that anti-Tac recognizes both receptor classes with similar affinity and that IL-2 inhibits Tac binding to both receptor classes in a competitive fashion. Tac antigens were purified from surface ^{125}I-labeled cells expressing high levels of high-affinity IL-2 receptors, but low levels of the low-affinity receptor class, and this preparation was compared with another pool of Tac antigens obtained from cells expressing the normal 10- to 20-fold excess of low-affinity IL-2 binding sites over high-affinity IL-2 receptors. Biochemical characterization by peptide mapping by limited proteolysis and two-dimensional gel analysis revealed that these distinct preparations of Tac antigens were indistinguishable. The data provide strong evidence that the 1000-fold affinity difference between the IL-2 receptor subsets is not due to molecular heterogeneity at the level of the primary amino acid sequence within the surface pool of Tac antigens.

Key words: interleukin-2 receptors/low-affinity receptors

Introduction

T lymphocyte cell cycle progression is regulated by the interaction of interleukin-2 (IL-2) and its specific membrane receptors (Morgan *et al.*, 1976; Möller, 1980; Cantrell and Smith, 1984; Robb *et al.*, 1981). IL-2 receptor expression is antigen dependent and upon removal of the immunostimulatory signal, IL-2 receptor levels decline progressively suggesting that the regulation of IL-2 receptors is a key parameter in the regulation of the immune response (Cantrell and Smith, 1983). Analysis of IL-2 receptor interactions has revealed at least two distinct affinity classes of IL-2 receptors (Robb *et al.*, 1984). The difference in dissociation constant (K_d) between these two receptor classes is of the order of 1000-fold (~10 pM versus ~10 nM) and the number of low-affinity receptors is normally 10- to 30-fold greater than that of the high-affinity receptors. The anti-Tac monoclonal antibody (mAb) has been used to purify the human IL-2 receptor from human T lymphotrophic virus I-infected leukemic T cells and cDNA encoding the protein has been isolated (Leonard *et al.*, 1982; Leonard *et al.*, 1984; Nikaido *et al.*, 1984). Using a co-transfection technique, stable expression of IL-2 receptor cDNA in mouse L cells has been reported but the expressed receptors exclusively exhibit a low apparent binding affinity for human IL-2 (Greene *et al.*, 1985).

The anti-Tac mAb has been widely used to analyse IL-2 receptors on human lymphocytes. Quantitative binding assays with radiolabeled IL-2 and anti-Tac indicate that activated T cells have 10-30 times more binding sites for the mAb than high-affinity sites for IL-2 (Robb *et al.*, 1984). As suggested by Robb *et al.*, a likely explanation for this difference between the two binding assays is that the majority of the anti-Tac binding sites represent the low-affinity IL-2 receptor. In this paper, a cellular system is described in which the level of anti-Tac binding sites can be manipulated after re-induction of optimal numbers of high-affinity IL-2 receptors on normal human T cell lines. It is shown that the low levels of Tac antigens correlate with very low levels of low-affinity IL-2 receptor expression. Moreover, almost half of all Tac antigens expressed on such cells consist of high-affinity IL-2 receptors and anti-Tac recognizes equally both high- and low-affinity IL-2 receptors.

The possibility of manipulating the IL-2 receptor composition, together with a mAb which recognizes both receptor classes, provides a means to analyse the molecular basis for high- versus low-affinity IL-2 binding. Thus, the pool of Tac antigens from cells with low levels of low-affinity IL-2 receptors was immunoaffinity purified and compared with the pool of Tac antigens prepared from comparable cells with the normal IL-2 receptor composition. These distinct pools of Tac antigens were compared both by peptide mapping by limited proteolysis and two-dimensional gel analysis. As the two preparations were indistinguishable by these criteria it is concluded that the gross affinity differences between high- and low-affinity IL-2 binding sites are not due to molecular heterogeneity at the level of the primary amino acid sequence within the cell surface pool of Tac antigens. Moreover, Tac antigens prepared from cells expressing distinct IL-2 receptor compositions also have the same isoelectric point (pI) and mol. wt. Thus, molecular heterogeneity at the level of heavy glycosylation is unlikely and the observed affinity difference seems rather to reflect conformational changes wtihin the receptor pool. Such conformational changes might be caused by a putative membrane protein non-covalently associated with a fraction of the Tac antigens.

Results

Manipulation of low-affinity IL-2 receptor expression

The development of a cellular system in which the level of Tac antigens can be manipulated was facilitated by the following observations: (i) high-affinity IL-2 receptors and Tac antigens have different turnover characteristics (Smith and Cantrell, 1985); (ii) high levels of Tac antigen receptor are strictly IL-2 dependent (Smith and Cantrell, 1985; Welte *et al.*, 1984; Reeves *et al.*, 1985; Reem and Yeh, 1984; Depper *et al.*, 1985); (iii) phorbol 12,13-dibutyrate (PBt_2) can induce homogeneous T cells to high-

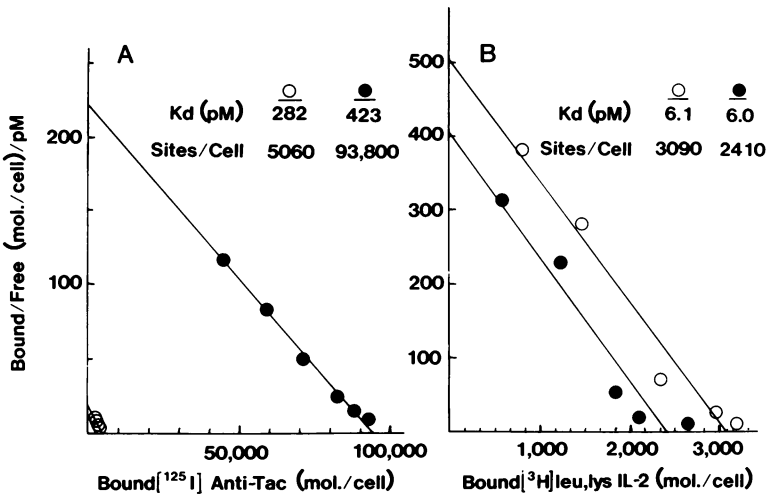

Fig. 1. Scatchard analysis of [^{3}H]leu, lys-IL-2 and [^{125}I]anti-Tac mAb binding. Long-term cultured T cells (14 days) were either IL-2-induced (closed symbols) or IL-2-deprived (open symbols) after 5 h stimulation with PBt_2 as described in Materials and methods. Equilibrium binding assays were performed with [^{125}I]anti-Tac **(panel A)** and [^{3}H]leu, lys-IL-2 **(panel B)** as described in Materials and methods.

affinity IL-2 receptor expression without inducing *in situ* IL-2 production (Smith and Cantrell, 1985) (data not shown) and was therefore used to reactivate IL-2 receptor expression on long-term cultured T cells. Accordingly, T cells obtained after 14 days of IL-2-dependent culture were proteolytically treated to remove residual Tac antigen expression, and thereafter stimulated with PBt_2 for 5 h. The high-affinity IL-2 receptor verus Tac antigen expression in these cells, 18 h after re-culture in IL-2-free medium, is shown in Figure 1 as Scatchard plots of binding data. The same figure also shows the effect of IL-2 treatment (140 pM) on parallel cultures of PBt_2-stimulated cells. The results clearly show that the expression of high-affinity IL-2 receptors is relatively independent of the ligand, while there are 10- to 20-fold more Tac antigens on cells that have been treated with IL-2 after PBt_2 stimulation.

Estimation of high-affinity IL-2 binding sites on IL-2-induced cells shown in Figure 1 was performed after 2 h of dissociation prior to equilibrium binding analysis. After this period of dissociation, IL-2-induced cells normally express 10–30% fewer high-affinity IL-2 binding sites as compared with IL-2-deprived cells which have been re-cultured in IL-2-free medium after PBt_2 stimulation.

Both the high- and low-affinity IL-2 receptors are recognized by anti-Tac mAb (Robb *et al.*, 1984). Thus, it is possible that the large amount of Tac antigens observed in IL-2-induced cells is due to IL-2-mediated enhancement of IL-2 low-affinity receptor expression. This possibility was directly investigated by performing [^{3}H]leu, lys-IL-2 binding assays using a high concentration of the ligand. The result from such a binding experiment (Figure 2) reveals strikingly different binding curves for IL-2-induced compared with IL-2-deprived cells. Thus, while IL-2-deprived cells show saturable binding at 50–100 pM (panel B), IL-2-induced cells show increasing amounts of binding which do not even saturate at 100 times higher concentrations (panel A). Scatchard analysis of the binding data (panels C and D) indicates two distinct receptor classes on IL-2-induced cells and the low-affinity class represents >90% of the total IL-2 binding to these cells. In contrast, IL-2-deprived cells express mainly high-affinity [^{3}H]leu, lys-binding sites and the low-affinity receptor class could not be conclusively demonstrated with the method used.

The relationship between the epitope recognized by anti-Tac and IL-2 high- and low-affinity binding sites

The possibility of manipulating IL-2 receptor composition, as outlined above, opens new routes to study the relationship between high- and low-affinity binding sites. Thus, it is possible to study whether anti-Tac binds to both the high- and low-affinity IL-2 receptor with the same affinity. In a first set of experiments the concentration dependence of IL-2 inhibition of [^{125}I]anti-Tac binding to each receptor class was studied. The result from such experiments (Figure 3, panel A) confirms that high concentrations of affinity-purified IL-2 almost completely inhibit [^{125}I]anti-Tac binding and a similar degree of inhibition by high concentrations of IL-2 is obtained with both IL-2-induced and IL-2-deprived cells. Most importantly, at IL-2 concentrations that only saturate high-affinity receptors, >50% of all [^{125}I]anti-Tac binding is inhibited in IL-2-deprived cells, while <5% inhibition is obtained in the IL-2-induced population which has a 10- to 20-fold higher ratio between high- and low-affinity IL-2 receptors (Figure 3, panel B). Extrapolation from the data shown in Figure 3 indicates that ~1000 times higher concentrations of IL-2 are required to inhibit 50% of anti-Tac binding to low-affinity sites as compared with inhibition of the high-affinity sites. These results confirm that anti-Tac binds to both IL-2 receptor classes and in addition these data indicate that approximately half of all molecules recognized by anti-Tac on IL-2-deprived cells are high-affinity IL-2 receptors.

To analyse further the relationship between IL-2 high- and low-affinity binding sites and the binding site of anti-Tac, equilibrium binding assays were performed. Graded concentrations (0.25–31 nM) of immunoaffinity-purified IL-2 were mixed with [^{125}I]-anti-Tac and binding at 4°C was determined using both IL-2-deprived and IL-2-induced cells. The binding curves of [^{125}I]anti-Tac in the presence of various concentrations of affinity-purified IL-2 (Figure 4, panels A and B) confirm IL-2-dependent inhibition of anti-Tac binding. Scatchard analysis of the binding data (panels C and D) shows that IL-2 inhibits [^{125}I]anti-Tac binding to both IL-2-deprived and IL-2-induced cells in a competitive fashion — i.e. IL-2 decreased the affinity of [^{125}I]anti-Tac binding rather than its maximal extent of binding at saturation. Moreover,

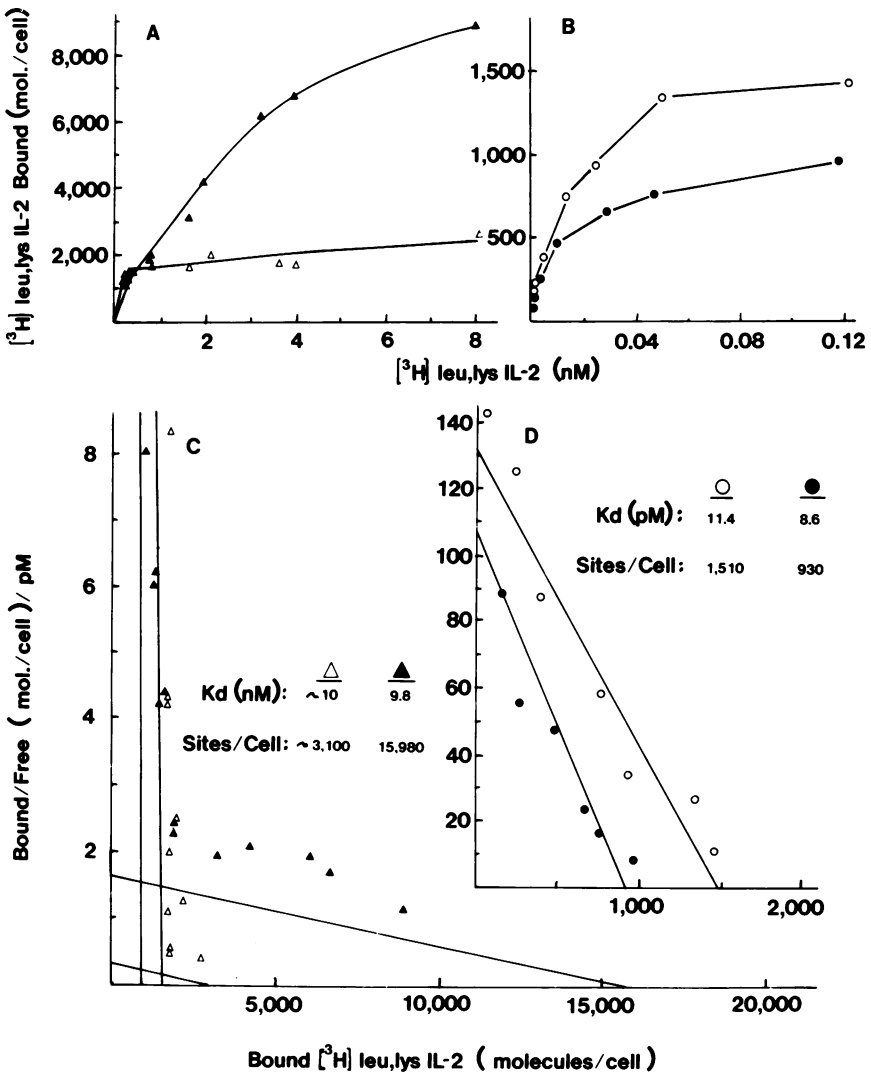

Fig. 2. Low-affinity binding of [^{3}H]leu, lys-IL-2 to IL-2-induced versus IL-2-deprived cells. Long-term cultured T cells were treated as described in Figure 1 and [^{3}H]leu, lys-IL-2 binding assays on IL-2-induced (closed symbols) and IL-2-deprived (open symbols) cells were performed as described in Materials and methods. **Panels A** and **B** show binding at high and low concentrations of [^{3}H]leu, lys-IL-2 and **panels C** and **D** show Scatchard analysis of the same data.

as can be anticipated by the large fraction of high-affinity IL-2 receptors in the total pool of Tac antigens on IL-2-deprived cells, concentrations of IL-2 (0.25−1 nM) that saturate high-affinity receptors caused a significant decrease in the apparent affinity of [^{125}I]anti-Tac binding to these cells. In contrast IL-2-induced cells were essentially unaffected by such low IL-2 concentrations.

IL-2-mediated inhibition of [^{125}I]anti-Tac binding was also determined at 37°C. Comparison of the apparent affinity of anti-Tac binding at 37 and 4°C shows a 4-fold decrease in binding affinity at the lower temperature and the calculated number of binding sites are also similarly decreased to about 60−80% of the sites detected at physiological temperatures (data not shown). However, there is no obvious reason why these dissimilarities between binding at 37 and 4°C should interfere with the interpretation of the results. At 37°C IL-2-mediated inhibition of [^{125}I]-anti-Tac binding to IL-2-deprived cells shows a clear-cut qualitative difference in inhibition patterns and this phenomenon was dependent on the dose of IL-2 used (Figure 5, panels A and C). Thus, Scatchard analysis of binding data (panel C) shows that 0.25−1.25 nM IL-2 inhibits [^{125}I]anti-Tac binding to IL-2-deprived cells in a non-competitive fashion — i.e. IL-2 causes a >30% decrease in the number of anti-Tac sites without significantly changing the apparent affinity. Moreover, high con-

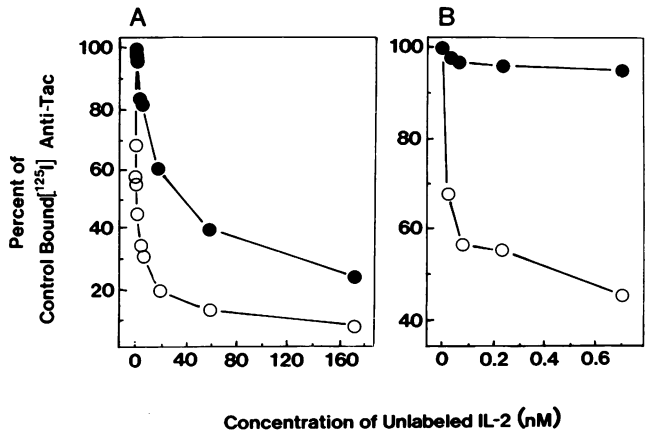

Fig. 3. IL-2-mediated inhibition of [^{125}I]anti-Tac binding on IL-2-induced versus IL-2-deprived cells. Long-term cultured T cells were treated as described in Figure 1 and IL-2-induced (closed symbols, 2100 high-affinity IL-2 receptors, 51 000 anti-Tac mAb binding sites) and IL-2-deprived cells (open symbols, 3200 high-affinity IL-2 receptors, 4100 anti-Tac mAb binding sites) were incubated for 20 min at 37°C in the presence of 1 nM [^{125}I]anti-Tac and the indicated concentration of affinity-purified IL-2. Cell-associated [^{125}I]anti-Tac was separated from free antibodies as described for equilibrium binding assays in Materials and methods.

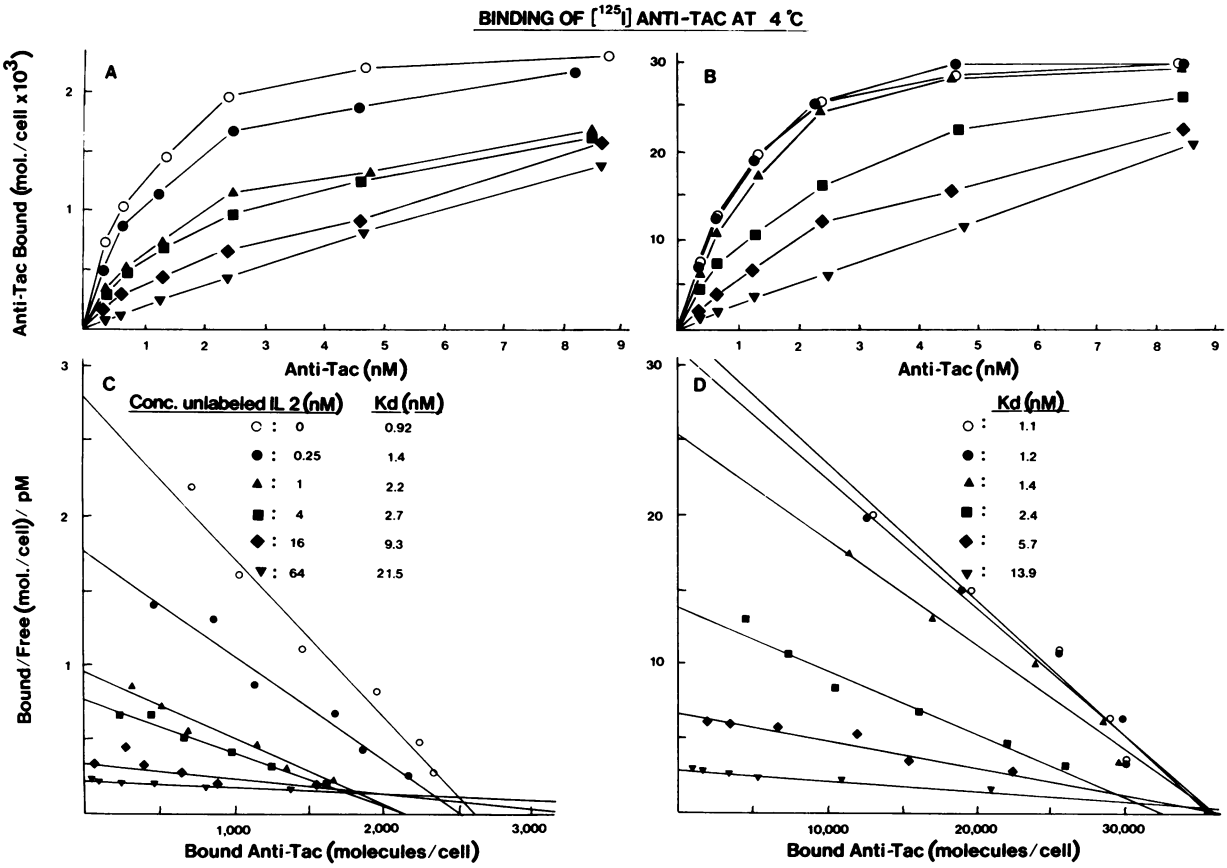

Fig. 4. Competitive IL-2-dependent inhibition of [^{125}I]anti-Tac binding at 4°C. Long-term cultured T cells were treated as described in Figure 1 and IL-2-deprived **(panels A** and **C)** and IL-2-induced **(panels B** and **D)** cells were incubated for 45 min at 4°C in the presence of the indicated concentrations of [^{125}I]anti-Tac and affinity-purified IL-2. Equilibrium binding assays were performed as described in Materials and methods and the lower panels show Scatchard conversion of the binding curves shown in the upper panels.

centrations (6.25–31.25 nM) of IL-2 do not decrease the number of anti-Tac sites below the level obtained with low concentrations, but rather inhibit specific anti-Tac binding in a competitive fashion. The result obtained with IL-2-induced cells from the same experiment (panels B and D), demonstrates that the phenomenon of an IL-2-dependent decrease in the total number of Tac antigens only becomes evident by using cells with a large fraction of high-affinity IL-2 receptors in their total pool of Tac antigens. Consequently, this phenomenon is not evident using IL-2-induced cells with the normal 1:20 ratio of high- and low-affinity IL-2 receptors.

Comparison of the apparent K_d of [^{125}I]anti-Tac binding to IL-2-deprived and IL-2-induced cells (Figure 5, insert), reveals that IL-2-mediated changes in [^{125}I]anti-Tac binding affinity at 37°C are independent of the relative fraction of high-affinity IL-2 receptors in the total pool of Tac antigens. A likely mechanistic explanation of IL-2-dependent non-competitive inhibition of [^{125}I]-anti-Tac binding is ligand-induced receptor internalization. This possibility is supported by the finding that the phenomenon does not occur if the experiment is performed at 4°C (Figure 4).

Biochemical analysis of the surface pool of Tac antigens on IL-2-induced and IL-2-deprived cells

The results above demonstrate that the anti-Tac mAb recognize both high- and low-affinity IL-2 receptors with a similar apparent affinity. This property of anti-Tac together with the possibility of manipulating low-affinity IL-2 receptor expression makes it feasible to use anti-Tac to immunoaffinity purify a receptor population containing defined ratios of high- and low-affinity binding sites. To distinguish biochemically the respective pools of Tac antigens expressed by IL-2-induced and IL-2-deprived cells, such cell populations were enzymatically surface iodinated and the cell lysates were absorbed to affigel-coupled anti-Tac mAb. After extensive washing the bound material was eluted and analysed on SDS–polyacrylamide gel electrophoresis (SDS–PAGE);a single band of 60 kd was obtained from both cell populations (Figure 6, lane A). As could be expected, ~5- to 10-fold more anti-Tac specific radioactivity was routinely recovered from IL-2- induced cells. A 40 kd species of the Tac antigens has been shown to represent a precursor form of the 60 kd protein (Leonard *et al.*, 1983; Wano *et al.*, 1984). The absence of this 40-kd species in the autoradiograph shown in Figure 6, lane A, can be considered as evidence that cells remained fully viable during the labeling procedure and consequently only Tac antigens detected on viable cells are iodinated. To compare the structure of high- versus low-affinity IL-2 receptors, the two pools of Tac antigens were digested with *Staphylococcus aureus* V8 protease or papain, two proteases with different recognition sites, at concentrations yielding partial digestion. As revealed by the peptide patterns in Figure 6 lanes B–E, the band pattern on the left side (IL-2-deprived cells) is indistinguishable from the band pattern on the right side (IL-2-induced cells). In addition, two-dimensional SDS–PAGE analysis of these Tac antigen preparations revealed a single spot with a pI between 5.5 and 4.8, with indistinguishable positions for both preparations of Tac antigens (Figure 7). Thus, by the criteria of one-dimensional peptide map-

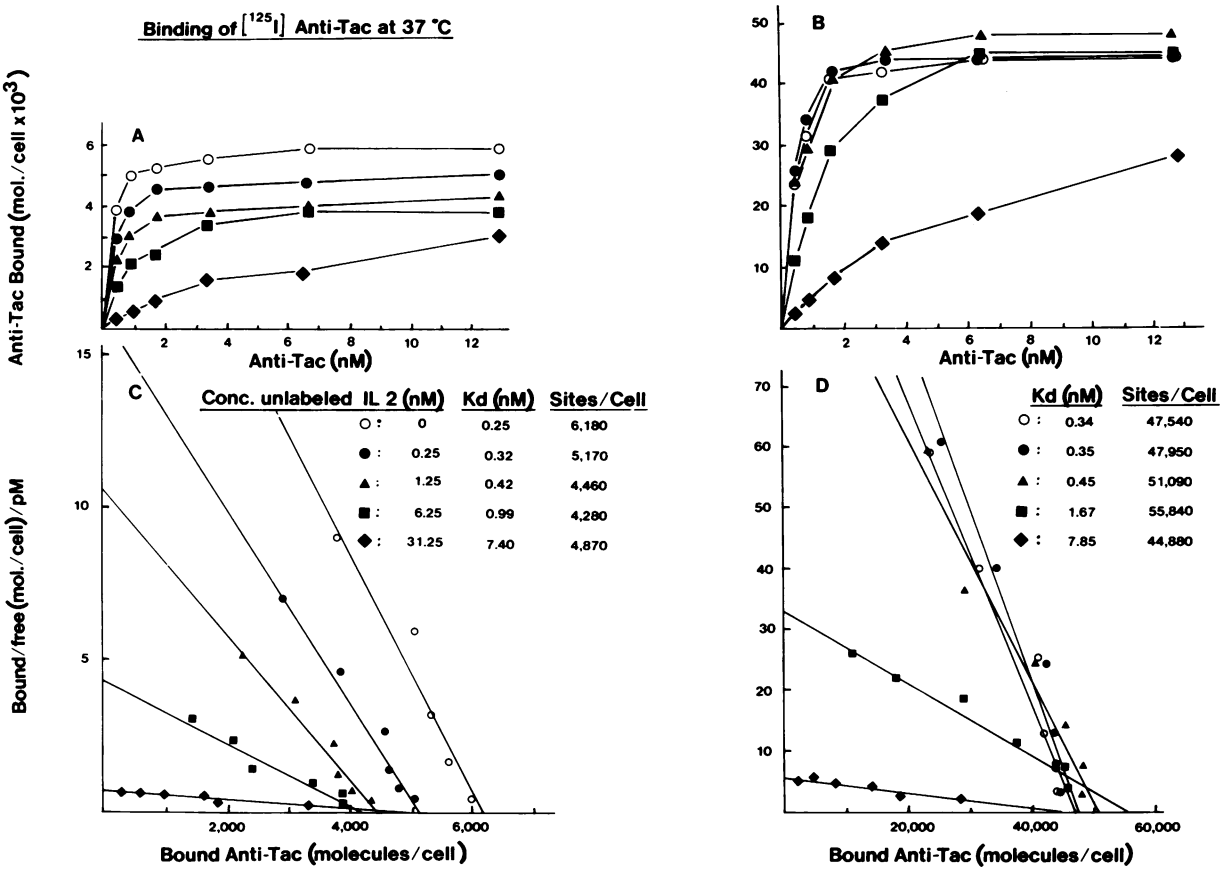

Fig. 5. IL-2-dependent inhibition of [^{125}I]anti-Tac binding at 37°C. The experiment was performed as described in Figure 4 but binding was performed during 20 min at 37°C. The symbols representing various concentrations of affinity-purified IL-2 and [^{125}I]anti-Tac are as indicated.

ping and two-dimensional SDS−PAGE analysis, high- and low-affinity IL-2 receptors, as defined by the protein chain recognized by anti-Tac, are biochemically indistinguishable.

Discussion

We have described a cellular system allowing extensive manipulation of high-affinity IL-2 receptor expression on normal human T cell lines. Optimal induction of expression of high-affinity IL-2 receptor can be obtained by stimulation with PBt_2 alone while maximal expression of low-affinity IL-2 receptors requires the synergistic action of both the phorbol ester and the ligand. IL-2-mediated enhancement of Tac antigen expression has been reported (Smith and Cantrell, 1985; Depper *et al.*, 1985). By comparing IL-2-induced with IL-2-deprived cell populations, the present study confirms that most, if not all, Tac antigens induced by IL-2 can bind the ligand, but the vast majority of all binding sites are the low-affinity type. Moreover, the optimal IL-2-dependent enhancement of anti-Tac binding sites coincides with optimal stimulation of T cell growth (Smith and Cantrell, 1985; Depper *et al.*, 1985), and in the present study both events were obtained with 140 pM IL-2 which is a concentration that only occupies a minor fraction of all low-affinity sites. This makes it likely that IL-2-dependent enhancement of low-affinity receptors is mediated via the high-affinity IL-2 receptor class, indicating a causal relationship between the two receptor classes.

The specific pattern of IL-2-mediated inhibition of [^{125}I]anti-Tac binding gave an approximate estimate of the relative fractions of high- and low-affinity IL-2 binding sites. The result in Figure 3 demonstrated that [^{125}I]anti-Tac binding to cells containing 3200 high-affinity sites in a pool of 4100 anti-Tac antigens could be >50% inhibited by <1 nM IL-2. Since the corresponding IL-2-induced cells expressed <5% of low-affinity IL-2 receptors in their total pool of Tac antigens, this cell population provided an internal control in both this and other experiments. Thus, the conclusions drawn from this study are all based on the use of two distinct, but still comparable, cell populations which have been treated to achieve a drastic difference in their IL-2 receptor composition. Scatchard analysis of IL-2-dependent inhibition of [^{125}I]anti-Tac binding at 37°C (Figure 5) was particularly revealing in separating different receptor classes. Thus, IL-2 inhibited the anti-receptor mAb binding to low-affinity binding sites in a non-competitive fashion while binding to low-affinity sites was inhibited in a competitive fashion.

The present study does not prove that the observed competitive inhibition of [^{125}I]anti-Tac binding is a competitive inhibition in the strict sense, but this does not alter the central argument that IL-2 inhibits [^{125}I]anti-Tac binding to IL-2 receptors in at least two distinct ways. Non-competitive inhibition of [^{125}I]anti-Tac binding to low-affinity binding sites at 37°C is most probably due to ligand-induced internalization of the high-affinity receptor since none of these phenomena are observed at 4°C (Figure 4). Subsequent analysis of receptor internalization in the presence and absence of the ligand supports this explanation (unpublished data).

A large difference in affinity between high- and low-affinity IL-2 receptors together with ligand-induced high-affinity receptor internalization made it possible to obtain a clear distinction between the receptor classes by using IL-2 to inhibit [^{125}I]anti-Tac binding. There are obvious technical difficulties in estimating low

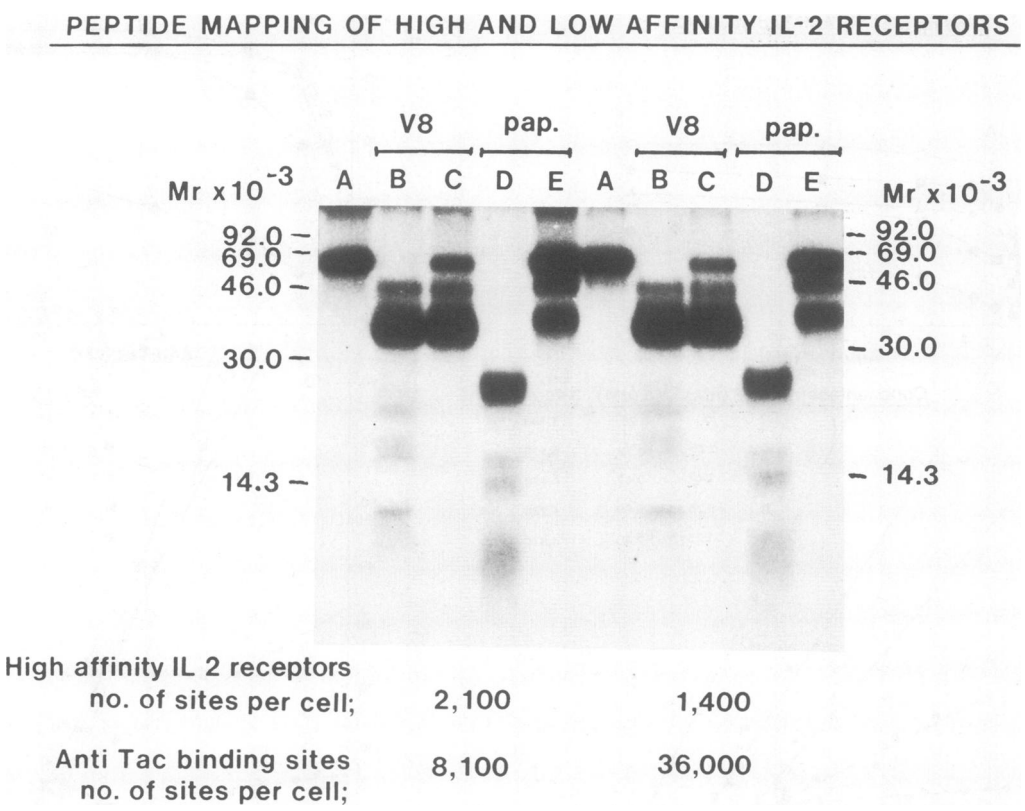

Fig. 6. Proteolytic digestion of Tac antigens from IL-2-deprived and IL-2-induced T cells. Long-term cultured T cells were treated as described in Figure 1 and IL-2-deprived (**A–E**, left side) and IL-2-induced (**A–E**, right side) cells were enzymatically surface iodinated and Tac antigens were immunoprecipitated with anti-Tac coupled to affigel as described in Materials and methods. Tac antigens were either undigested **(lane A)** or digested with 7.5 μg **(lanes B** and **D)**, 0.25 μg **(lanes C** and **E)** *S. aureus* V8 protease or papain as indicated. The numbers of high-affinity IL-2 receptors and anti-Tac mAb binding sites on the respective cell populations are shown under the autoradiograph of a dried 18% SDS–PAGE slab gel.

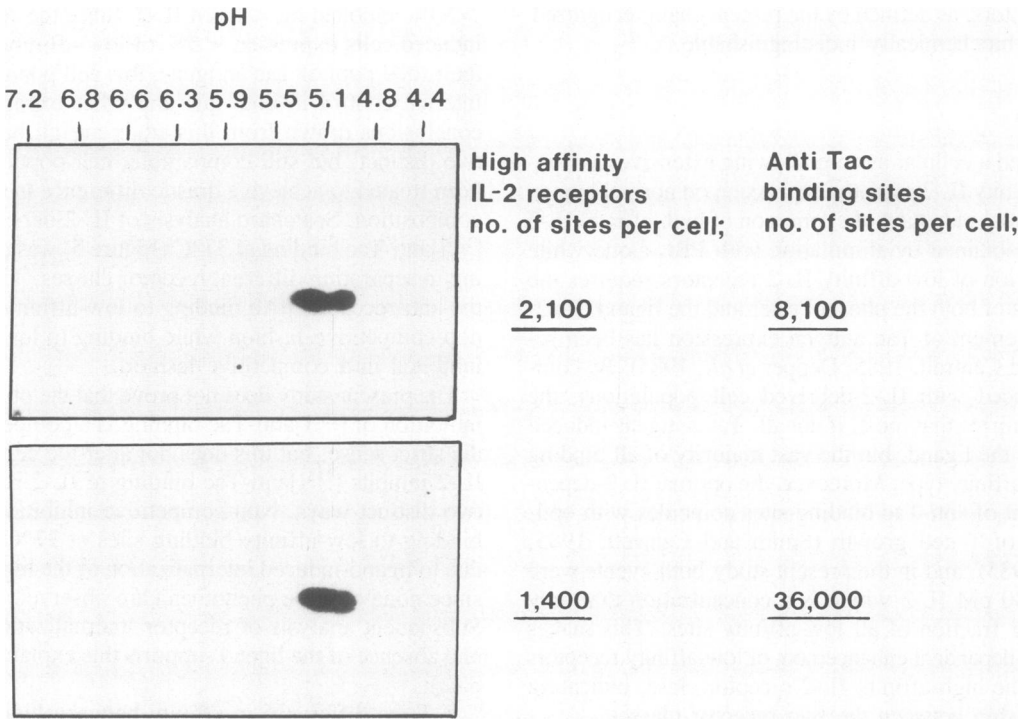

Fig. 7. Two-dimensional SDS–PAGE analysis of Tac antigens from IL-2-deprived and IL-2-induced cells. Autoradiograms from two-dimensional electophoresis of the same Tac preparations used in Figure 6. IL-2-deprived cells: **upper panel**. IL-2-induced cells: **lower panel**.

levels of low-affinity IL-2 binding sites with [^{3}H]leu, lys-IL-2 and directly to compare estimates of the number of sites obtained using two different radiolabeled probes might give misleading results. Thus, the specific activity of the ligand and the antibody is estimated by different methods and unexpected cross-reactivities by the respective probes cannot be excluded. However, using IL-2, that is *a priori* specific for IL-2 receptors of any affinity class, to inhibit binding of an IL-2 receptor-specific mAb provides in the present system an estimate of the minimal amount of high-affinity IL-2 receptors in the total surface pool of Tac-reactive proteins. The availability of two distinct but comparable cell populations, with defined large differences in their IL-2 receptor composition, made it possible to analyse biochemically the structural basis for gross affinity differences. As the receptor composition was defined at the level of binding to viable cells it was important to ensure that only surface-associated receptors are analysed. In this study enzymatic surface labeling with iodine was used to avoid interference by an unknown intracellular pool of Tac antigens. By the criteria of cell viability after the labeling procedure and the absence of the 40 kd species, which is observed after Tac precipitation of biosynthetically labeled cells (Leonard *et al.*, 1983; Wano *et al.*, 1984), it seems likely that the labeled Tac antigens analysed herein represent the same pool of Tac antigens analysed by binding experiments. As the radiolabeled receptor populations from IL-2-deprived and IL-2-induced cell populations were indistinguishable by the methods used in the present study, it seems highly unlikely that the large affinity differences between the two receptor classes is due to molecular heterogeneity at the level of the primary amino acid sequence in the surface pool of anti-Tac reactive proteins.

Identification of distinct receptor complexes that account for high- and low-affinity glucagon binding has previously been reported and these receptor complexes seem to differ in glycosylation (Mason and Tager, 1985). The Tac antigen is heavily glycosylated in cells with the normal IL-2 receptor composition and post-translational modifications have a strong influence on both pI and mol. wt (Leonard *et al.*, 1983; Wang *et al.*, 1984). Although the present study has not excluded minor differences in post-translational modifications, it shows that surface-associated Tac antigens prepared from cells with distinct IL-2 receptor compositions have indistinguishable pI and mol. wt. Thus, differential post-translational processing of high- and low-affinity IL-2 receptors sems unlikely but further analysis is required to rule out totally this possibility.

Molecular cloning of the Tac antigen has formally proved that this protein is an IL-2 binding protein (Leonard *et al.*, 1984; Nikaido *et al.*, 1984). However, stable transfectants of mouse L cells, expressing a Tac antigen cDNA, expressed the low-affinity but not the high-affinity form of the IL-2 receptor (Greene *et al.*, 1985). Hatakeyama *et al.* (1985) have reported that both high- and low-affinity IL-2 binding on a murine T lymphoma after transfection with Tac antigen cDNA. The reported kd of the high-affinity counterpart was 160–220 pM and the affinity difference between the receptor subsets was only 10-fold. Thus, the binding characteristics of these transfectants were quite different from those of the one observed in the present study and in previous reports by others (Robb *et al.*, 1981). However, in a recent report it was demonstrated that a murine IL-2-dependent T cell clone, after transfection with Tac antigen cDNA, responds to human but not murine IL-2 in the presence of a mAb specific for the murine IL-2 receptor (Kondo *et al.*, 1986). Although no direct binding could be performed to analyse specifically the human high-affinity IL-2 receptor, this report strongly suggested that a Tac antigen cDNA can direct the synthesis of both high- and low-affinity receptors.

Conformational changes of a single receptor complex have been postulated in other ligand–receptor systems to be responsible for corresponding affinity changes (Buxser *et al.*, 1983a,b; Grob and Bothwell, 1983). The present study supports the concept that high- and low-affinity IL-2 receptors are identical proteins as defined by the Tac antigen and it follows that conformational changes within an IL-2 receptor complex might create affinity changes. Binding experiments performed at 4°C revealed that IL-2 inhibits [^{125}I]anti-Tac binding to both receptor classes in a competitive fashion (Figure 4), although the high-affinity receptor class was more sensitive to inhibition, and it is therefore likely that IL-2 competes with the mAb for the same binding sites on both receptor classes. A recent report by Lowenthal *et al.* (1985) supports the notion that conformational changes may result in a drastic affinity change. Thus, they have isolated a mAb specific for the murine IL-2 receptor which appears to inhibit IL-2 binding without interacting with the IL-2-specific binding site on the receptor, but rather by increasing the rate of IL-2 dissociation. Thus, it is possible that a protein, non-covalently associated with the IL-2 receptor, can have a great influence on the apparent affinity.

Materials and methods

Cell cultures

Human peripheral blood mononuclear cells, isolated by Ficoll–Hypaque discontinuous gradient centrifugation, were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) (Sterile Systems, Inc., Logan, UT), 50 U/ml penicillin, 300 μg/ ml L-glutamine, 2.5 μg/ml insulin, 2.5 μg/ml transferrin and 2.5 ng/ml selanous acid in a humidified atmosphere of 5% CO_2 in air. PBt_2 (Sigma) was used to re-induce IL-2 receptor expression.

Long-term T cell lines were initiated by 3 days of OKT-3 stimulation followed by pronase treatment (Sigma, 200 μg/ml, 30 min at 37°C) and cells were re-cultured in medium containing IL-2 (140 pM). After 12–18 days of IL-2-dependent growth these cells were expanded 100- to 200-fold and 90% were in the G_0/G_1 phase of the cell cycle. Expression of optimal levels of high- and low-affinity receptors was achieved by a 5-h treatment with PBt_2 (20 ng/ml) followed by re-cultivation in the presence of affinity-purified IL-2 (140 pM) for 20–24 h. Expression of optimal levels of high-affinity IL-2 receptors, without re-induction of the low-affinity receptor class, was obtained by proteolytic removal of all surface-bound IL-2 and its receptor followed by a 5 h treatment with PBt_2, After re-cultivation in IL-2-free medium for 16–20 h, these cells were used for experiments and will be referred to as IL-2-deprived cells, while cells stimulated with IL-2 after PBt_2 stimulation will be referred to as IL-2-induced cells.

Production and purification of IL-2

Jurkat subclone 6.8b was kindly provided by Dr John Lowenthal and induction to IL-2 production was performed as previously described (Robb *et al.*, 1981). Unlabeled and biosynthetically radiolabeled [^{3}H]leu, lys-IL-2 were purified by NH_3SO_4 precipitation followed by absorption to an immunoaffinity column constructed with an IgG_{2a} mAb to IL-2 (designated DMS-3) (Smith *et al.*, 1983). Washing steps and elution were performed as described by Smith *et al.* (1985) and the eluted material comprised a single M_r 15 500 protein as far as could be determined by SDS–PAGE (data not shown).

The specific activity of the radiolabeled IL-2 was determined by relating radioactivity to biological activity. Biological activity was correlated with the protein mass by using a reference reagent of Jurkat IL-2 (specific activity: 13.1×10^6 reference units/mg) kindly supplied by Biological Response Modifiers Program, Biological Resources Branch, NCI-FCRF Frederick, MD. The biological assay was performed by using the IL-2-dependent cytolytic T cell clone CTLL as described by Gillis *et al.* (1978). The specific activity of [^{3}H]leu, lys-IL-2 was calculated as 95 400 d.p.m./pmol.

IL-2 and anti-Tac binding assays

[^{3}H]Leu, lys-IL-2 was prepared and purified as described above. Anti-Tac mAb (a gift from Dr T.Waldmann) was purified from ascites fluid by NH_3SO_4 precipitation and protein A–Sepharose (Pharmacia) affinity adsorption and radio-

labeled using Enzymobeads (Bio-Rad) (specific activity 2 × 10^5 c.p.m./pmol).

All cells were prepared by binding assays by selection of viable cells of Ficoll–Hypaque discontinuous gradient centrifugation, followed by incubation at 37°C in IL-2-free medium for 2 h. For equilibrium binding analysis, serial dilutions of [^{3}H]leu, lys-IL-2 or [^{125}I]anti-Tac mAb were incubated with 2–20 × 10^5 cells (0.2 ml) for 20 min at 37°C or 45 min at 4°C. Cell-bound and free radioactivity was determined after separation on a two-step gradient consisting of an upper layer (0.15 ml) of 84% silicone oil and 15% paraffin oil and a lower layer (0.1 ml) of FCS containing 10% sucrose. The number of binding sites per cell was determined by Scatchard analysis after subtraction of non-saturable binding, as detected in the presence of a 100 M excess of unlabeled ligand. Calculations were made by assuming monovalent antibody binding to the Tac antigen and slopes were determined by linear regression analysis.

Immunoprecipitation and analysis of Tac antigens

Cell surface iodination of cells was performed with [^{125}I]NaI (New England Nuclear) and catalysed by lactoperoxidase. To 100 × 10^6 cells suspended in 5 ml of phosphate-buffered saline were added successively 50 μl of glucose (0.5 M), 25 μl of NaI (0.5 mM), 50 μl of lactoperoxidase (2 mg/ml), 8 mCi (1 Ci = 3.7 × 10^{10} Bq) of [^{125}I]NaI and 100 μl of glucose oxidase (750 milliunits/ml). This mixture was incubated fro 15 min at room temperature and then 500 μl of NaI (1 M) was added. Cells were washed and the pellet was lysed in 1 ml of 1:10 diluted radioimmunoprecipitation assay (RIPA) stock solution containing 1% Triton X-100 and 0.15 M NaCl (RIPA stock solution: 100 mM NaH_2PO_4/1 mM phenylmethyl sulfonyl fluoride (PMSF)/10 mM EDTA/10 mM NaF, pH 7.2). The suspension was centrifuged for 5 min at 9000 *g* and the resulting supernatant was desalted on Sephadex G25 and precleared with normal mouse IgG and protein A–Sepharose (Pharmacia, Uppsala, Sweden). Cleared lysates were incubated with 80 μl packed affigel coupled to anti-Tac mAb for 60 min at 4°C. The resulting precipitate was subsequently washed three times wtih 1:10 diluted RIPA solution containing 1 M NaCl, seven times in 1:10 diluted RIPA solution containing 1% Triton X-100 and 1% deoxycholate sodium salt and finally twice in 10 mM Tris–HCl pH 7.5. The precipitate was eluted from the gel by boiling in a buffer containing Tris–HCl pH 6.8, 0.5% SDS, 10% glycerol. The immunoprecipitation efficiency was 0.34 and 0.07% using lysates from IL-2- induced and IL-2-deprived cells, respectively.

Peptide mapping by limited proteolysis was performed according to the protocol of Cleveland *et al.* (1977). Samples were resuspended in gel buffer [0.125 M Tris–HCl pH 6.8, containing 10% (v/v) glycerol, 3% SDS and 10% (v/v) 2-mercaptoethanol] and boiled for 5 min. SDS–PAGE was performed on an 18% polyacrylamide vertical gel for 4 h and at 100 V according to the Laemmli procedure (Laemmli, 1970). Molecular masses were estimated from the mobilities of ^{14}C-labeled markers (Amersham) (myosin, 200 kd; phosphorylase, 92.5 kd; bovine serum albumin, 69.0 kd; ovalbumin, 46.0 kd; carbonic anhydrase, 30.0 kd; lysozyme, 14.3 kd). Gels were dried onto filter papers and autoradiograms were prepared by exposure of dried gels to X-ray film.

Two-dimensional electrophoresis was carried out according to the procedure described by O'Farrell (1975). First-dimension isoelectric focusing contained 1.2% ampholines pH 4–6.5 and 1.2% ampholines pH 5–8. Second-dimension SDS–PAGE contained 9% acrylamide and 0.8% bis-acrylamide.

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