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Involvement of dipeptidyl peptidase IV in an *in vivo* immune response

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SUMMARY

Dipeptidyl peptidase IV (DP IV) is a serine protease that selectively cleaves X-Pro dipeptides from polypeptides and proteins. Among blood cells, this enzyme occurs preferentially on the surface of $CD4^+$ T cells and the amount of enzyme activity increases with T cell activation. In previous work, two potent and specific peptidyl-boronic acid inhibitors of DP IV, Ala-boroPro and Pro-boroPro, were synthesized and Pro-boroPro was shown to suppress antigen-specific proliferative responses of T cells *in vitro*. In this study, we tested the *in vivo* effects of these inhibitors. Subcutaneous injection of Ala-boroPro or Pro-boroPro into BALB/c mice inhibited DP IV activity in serum and spleen cell suspensions. Repeated injections of more than 10 μg of Ala-boroPro or Pro-boroPro at 12 h intervals maintained *in vivo* DP IV activity at less than 30% of the normal level. Repeated injections of the inhibitors during the primary, secondary or tertiary immune response to bovine serum albumin (BSA) reduced anti-BSA antibody production. Without inhibitor, immunization with BSA was followed by a temporary decrease in serum DP IV activity and then by enhanced serum enzyme activity after several days. These results provide the first direct evidence that DP IV plays an important role in immune response *in vivo*.

Keywords dipeptidyl peptidase dipeptidyl peptidase IV protease inhibitor peptide boronic acid serine protease CD 26 accessory molecule antibody production

INTRODUCTION

Dipeptidyl peptidase IV (DP IV; EC 3.4.14.5) was first discovered in rat liver and kidney [1], and subsequently was demonstrated in salivary gland [2], small intestine [3], serum [4] and blood cells [5–7]. This enzyme preferentially hydrolyses a peptide bond on the carboxyl side of proline when proline is the penultimate residue from the free amino terminus, resulting in the release of an X-Pro dipeptide where X can be any amino acid [8]. Among blood cells, DP IV activity occurs on E-rosette-forming cells of normal individuals [5], and on some $CD4^+$ leukaemic cells [6]. Cytofluorometric analysis has revealed that most DP IV^+ cells are $CD4^+$, while a small fraction of the DP IV^+ cells belongs to the $CD8^+$ population [9].

Experiments with enzyme inhibitors indicate that this enzyme plays a role in *in vitro* immune responses. Two DP IV inhibitors, Ala-boroPro and Pro-boroPro, which have been used in our studies, are dipeptides containing the α-amino acid analog of proline (boroPro) as the C-terminal amino acid. They exhibit kinetic properties consistent with the mechanism for slow-binding inhibition and produce more stable enzyme-inhibitor complexes than do simple competitive inhibitors [10,11]. Inhibition of DP IV activity by Pro-boroPro leads to suppression of antigen-induced but not mitogen-induced T cell proliferation *in vitro* [11]. With higher concentrations of less potent inhibitors of DP IV or with polyclonal anti-DP IV antibodies, some investigators have observed suppression of mitogen-induced immune responses also [9,12–14]. In addition, mitogen-stimulated T cells in culture show an increase in DP IV activity [15,16].

This study is focused on the role of DP IV *in vivo*. We tested the effect of Ala-boroPro and Pro-boroPro on enzyme activity in serum and in lymphocyte suspensions and on an antigen-specific immune response.

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MATERIALS AND METHODS

Inhibitors of DP IV

Dipeptides containing boroPro as the C-terminal amino acid, Ala-boroPro and Pro-boroPro, were synthesized as described previously [11]. These inhibitors were dissolved in saline, sterilized with Millex-HA filter units (Millipore, Bedford, MA) and kept at -80°C until use.

Treatment of mice

From 5 to 8 week old female BALB/c mice were obtained from Taconic (Germantown, NY). Sterile inhibitors of DP IV were injected subcutaneously and blood samples were collected from the tail artery. The frequency of bleeding was limited to maintain the haematocrit higher than 30%. Serum or plasma from heparinized samples was separated and kept at −80°C until use. For preparation of spleen cell suspensions, erythrocytes were removed by lysis with Tris-NH_4Cl (17 mM Tris, 144 mM NH_4Cl, pH 7·2) and cells were washed with PBS, and then suspended in HEPES-saline (25 mM HEPES, 140 mM NaCl, pH 7·8). Viability of the cells, determined by trypan blue dye exclusion, was >90% during the assay. In experiments on anti-bovine serum albumin (BSA) responses, BSA (Sigma, St Louis, MO) was dissolved in saline, filtered with Millex-HA filter units and injected intraperitoneally with or without Ribi Adjuvant (RIBI, Hamilton, MT). Dosages of BSA are given in the text for individual experiments.

Assay for DP IV

Ten microlitres of serum or plasma were diluted, or 2×10^6 spleen cells were suspended in 3 ml of HEPES-saline and incubated with a fluorogenic substrate Ala-Pro-AFC (7-amino-4-trifluoromethyl coumarin; Enzyme Systems Products, Dublin, CA) in the cuvette of an LS-5B Luminescence Spectrometer (Perkin-Elmer, Beaconsfield, UK). One unit of DP IV activity was defined as the amount of enzyme catalysing the formation of 1 μmole/min of AFC at 25°C. The concentration of substrate was 13·3 μM unless indicated otherwise.

Assay for anti-BSA antibody

Anti-BSA antibody activity in immunized mouse sera was measured by ELISA. Immulon 2 microtitre plates (Dynatech, Chantilly, VA) were coated with 200 μg/ml of BSA in Tris-buffered saline (25 mM Tris, 140 mM NaCl, pH 7·4; TBS), washed with TBS, and blocked with TBS containing 1% gelatin. Serum samples were serially diluted with TBS containing 0·5% gelatin and incubated on the microtitre plates for 90 min. Bound antibodies were detected by anti-mouse IgG (γ chain specific)-alkaline phosphatase conjugate (Sigma) or anti-mouse IgM (μ chain specific)-alkaline phosphatase conjugate (Sigma) and *p*-nitrophenyl phosphate as a substrate. The amount of anti-BSA antibody was determined by comparison with a standard anti-BSA antibody purified by BSA-Sepharose affinity column chromatography.

Assay for total IgG and IgM

Total IgG and IgM in mouse sera were measured by radial immunodiffusion using anti-mouse IgG (γ chain specific) and anti-mouse IgM (μ chain specific) antibodies (Sigma). The standard purified mouse IgG and IgM were purchased from Zymed (San Francisco, CA).

RESULTS

Kinetics of serum DP IV

For comparison of serum enzyme with purified DP IV, untreated BALB/c mouse serum was incubated with several concentrations of the fluorogenic substrate Ala-Pro-AFC. The serum enzyme has a K_M of 8·4 μM for this substrate (Fig. 1), a value that is comparable to that of purified porcine enzyme (unpublished observation).

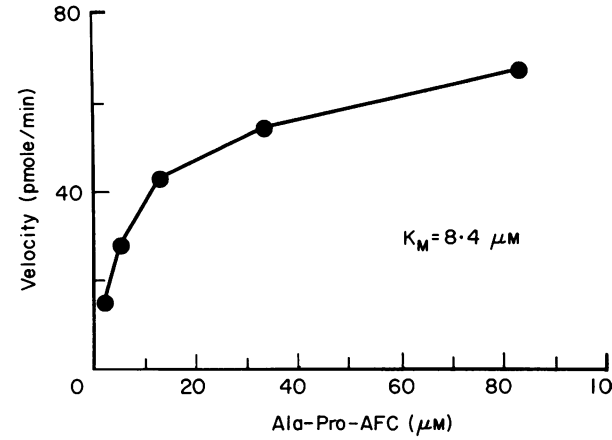

Fig. 1. Kinetics of serum dipeptidyl peptidase IV (DP IV). DP IV activity in BALB/c mouse serum was measured using various concentrations of Ala-Pro-AFC.

In vivo *effect of the inhibitors on DP IV*

Previous studies have shown that Ala-boroPro and Pro-boroPro are potent inhibitors of DP IV [11]. For the first *in vivo* trials, we tested the effect on serum enzyme activity of a single subcutaneous injection of these inhibitors. Both Ala-boroPro and Pro-boroPro inhibited DP IV activity *in vivo*. Two hours after a subcutaneous injection of 1, 10 or 100 μg of Ala-boroPro into BALB/c mice, serum DP IV activity was reduced to 28, 11 or 8·7% of normal, respectively, and it recovered to 78, 57 or 54%, respectively, by 23 h after the injection (Fig. 2a). Similarly, injection of 1, 10 or 100 μg of Pro-boroPro reduced serum DP IV activity to 52, 23 or 9·5% of normal, respectively, 2 h after the injection, and activity recovered to 88, 73 or 57% of normal, respectively, 23 h after the injection (Fig. 2b). Pro-boroPro was slightly less effective than Ala-boroPro at the low dose, but there was no significant difference between them at the high dose. Since free Pro-boroPro is more stable in aqueous solution [11], most of the following experiments were performed using Pro-boroPro.

To test the effect of consecutive injections, 10 or 100 μg of Pro-boroPro was injected four times at 12 h intervals. Consecutive injections of 10 μg of Pro-boroPro led to more inhibition of serum DP IV than a single injection of the same dosage. However, consecutive injections of 100 μg of Pro-boroPro did not exert more effect than a single injection. Doses of 10 μg and 100 μg of Pro-boroPro, administered repeatedly at 12 h intervals, had almost the same effect (Fig. 3).

To determine whether injected inhibitors can inhibit DP IV activity on lymphocyte surfaces, mice were killed 1 h after a subcutaneous injection of Pro-boroPro and DP IV activities in plasma and spleen cell suspensions were measured. Although plasma was used instead of serum in this experiment, we had confirmed that there is no substantial difference in DP IV activity of serum and plasma. Twenty micrograms of Pro-boroPro inhibited DP IV activity in a spleen cell suspension by 72% while it inhibited the plasma DP IV by 90% (Table 1). A higher dose of Pro-boroPro inhibited more DP IV activity in plasma and the cell suspension; however, residual activity in the

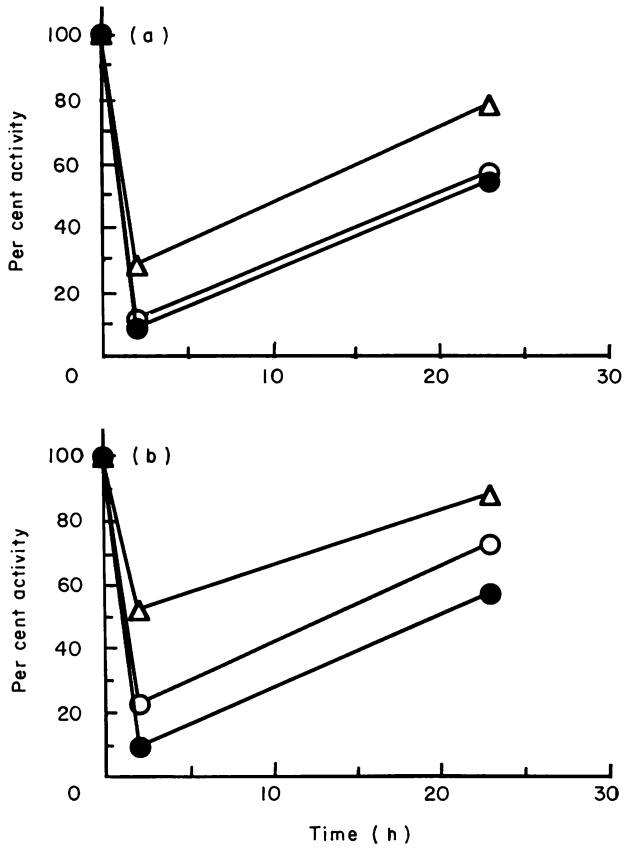

Fig. 2. *In vivo* effect of dipeptidyl peptidase IV (DP IV) inhibitors on serum DP IV. Serum DP IV activity was measured 2 and 23 h after a single subcutaneous injection of 1 (△), 10 (○) or 100 (●) μg of Ala-boroPro (a) or Pro-boroPro (b). Data show the mean value for sera from two mice.

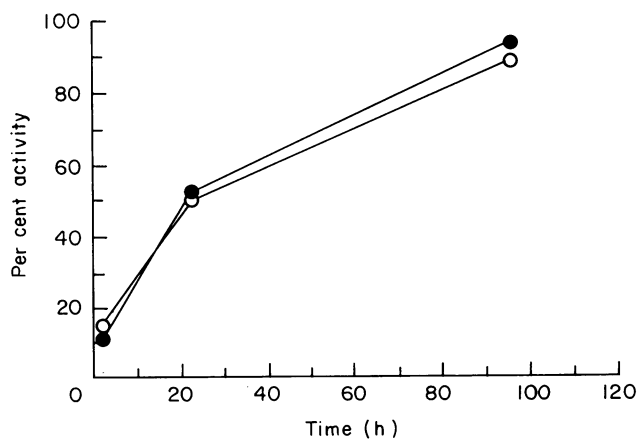

Fig. 3. Effect of consecutive injections of Pro-boroPro on serum dipeptidyl peptidase IV (DP IV). Ten (○) or 100 (●) μg of Pro-boroPro was injected four times at 12 h intervals. Serum DP IV activity was measured 2, 23, and 96 h after the last injection. Data show the mean value for sera from two mice.

Table 1. Effect of Pro-boroPro on dipeptidyl peptidase IV (DP IV) activity *in vivo*

Inhibitor (μg)	DP IV activity	
	Plasma (U/*l*)	Cell ($\times 10^{-12}$ U/cell)
0	4·16	22·6
20	0·40 (9·6%)	6·4 (28%)
40	0·28 (6·7%)	6·2 (27%)
80	0·16 (3·8%)	4·6 (20%)
160	0·08 (1·9%)	3·0 (13%)

DP IV activity in plasma and spleen cell suspensions of BALB/c mice was measured 1 h after the subcutaneous injection of Pro-boroPro. The values in parentheses indicate the percentage of residual enzyme activity in samples from treated animals.

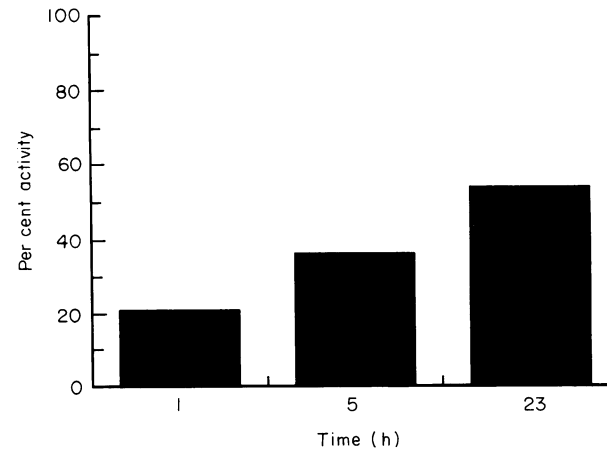

Fig. 4. *In vivo* recovery of cellular dipeptidyl peptidase IV (DP IV). DP IV activity in spleen cell suspensions was measured 1, 5 and 23 h after the injection of 50 μg of Pro-boroPro. Data show the mean value for cell suspensions from two mice.

cell suspension was always higher than that in plasma. Thirteen per cent of the cellular DP IV activity remained after injection of 160 μg of Pro-boroPro.

Figure 4 shows the *in vivo* recovery of cellular DP IV activity after subcutaneous injection of Pro-boroPro. In this experiment, mice were killed 1, 5 or 23 h after the injection of 50 μg of Pro-boroPro and DP IV activity in a spleen cell suspension was compared with that of untreated mice. Similar to the recovery of serum DP IV (Fig. 2), cellular DP IV activity recovered with time, and by 23 h after the injection it reached 54% of the activity of cells from untreated mice.

Effect of antigen stimulation on serum and cellular DP IV

In vitro studies have shown that T cell surface DP IV increases in accordance with T cell activation by mitogens [15,16]. We followed the level of serum and cellular DP IV during an antigen-induced immune response. Two days after immuniza-

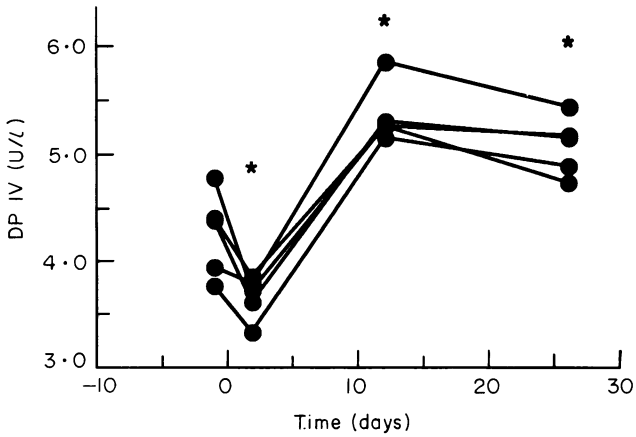

Fig. 5. Effect of antigen stimulation on dipeptidyl peptidase IV (DP IV) activity. Each line shows serum DP IV activity in five individual mice immunized with 100 μg of BSA with adjuvant. Data are representative of three separate experiments. *, Significantly different from pre-immune level ($P < 0.05$, Wilcoxon signed rank test).

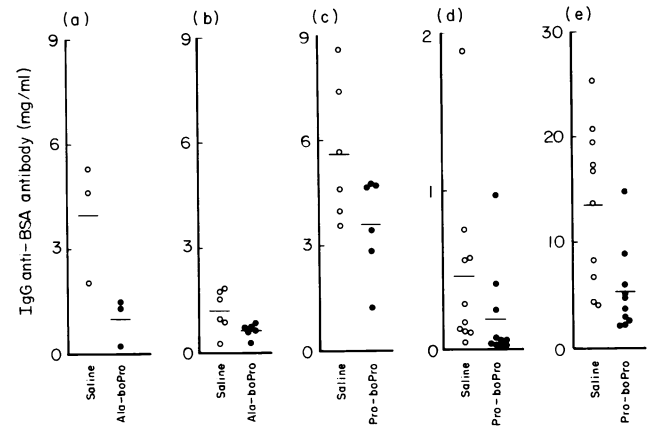

Fig. 6. Effect of dipeptidyl peptidase IV (DP IV) inhibitors on antibody production. Each circle shows the level of IgG anti-BSA antibody in individual mouse given the inhibitor (●) or saline for the controls (○) following immunization with BSA. In all of these experiments the differences between treated and control groups were statistically significant ($P < 0.05$, Mann-Whitney *U*-test). Horizontal bar represents the mean value. See text for details.

tion with BSA (day 2), all immunized mice had a serum DP IV activity lower than the pre-immune level (Fig. 5). Afterward, it rose to above the pre-immune level by day 12 and remained higher than the pre-immune level on day 26. We observed that this pattern of a post-immunization drop followed by a rise in serum enzyme activity occurred in three separate experiments performed with different bleeding schedules.

In another experiment mice were killed after immunization to measure DP IV activity in the spleen cell suspension. Two days after immunization, while serum DP IV was lower, cellular DP IV was higher by 12% than in unimmunized control mice, indicating that T cells had already been activated at that time (data not shown).

Effect of DP IV inhibitors on immune response

Since we confirmed that subcutaneously injected Ala-boroPro and Pro-boroPro inhibit DP IV in serum and spleen cell suspensions, we administered these inhibitors to mice immunized with BSA to determine the effect on antibody production. Subcutaneous injections of DP IV inhibitor or sterile saline for the control were started 1 h after (experiment 1) or 1 h before (experiments 2–4) immunization and repeated 2–3 times a day for 6–14 days during the immune response.

In experiment 1, six mice were immunized with 50 μg of BSA with adjuvant on days 0 and 21, and three of them were treated with Ala-boroPro (20 μg/day) only during the secondary response. All three treated mice showed lower IgG anti-BSA antibodies than the control group on day 28 (Fig. 6a). In experiment 2, six mice for each group were immunized with 10 μg of BSA with adjuvant on days 0 and 18, treated with Ala-boroPro (20–30 μg/day) during the primary response and with Pro-boroPro (40–60 μg/day) during the secondary response. The mean values of IgG anti-BSA antibody in inhibitor-treated animals in the primary (day 10, Fig. 6b) and secondary (day 24, Fig. 6c) responses were 0·63 and 3·59 mg/ml, while in control animals receiving saline instead of inhibitor the values were 1·20 and 5·62 mg/ml, respectively.

In experiment 3, 10 mice for each group were immunized with 100 μg of BSA on day 0, 18 and 28, and for the treatment Pro-boroPro (40–120 μg/day) was given after primary, secondary and tertiary immunizations. Both treated and control groups produced lower amounts of anti-BSA antibodies than other experiments because antigen was given without adjuvant in this case. During the very low primary and secondary responses, the difference between the two groups was not obvious, but after tertiary immunization, the treated group showed significantly lower IgG anti-BSA antibody levels than the control group on day 35 (0·19 *versus* 0·46 mg/ml, Fig. 6d); seven out of 10 mice in this group showed virtually no response. Fig. 6e (experiment 4) shows anti-BSA antibody on day 35 in mice repeatedly immunized with 50 μg of BSA with adjuvant (on days 0, 14 and 28). Treatment with Pro-boroPro (40–60 μg/day) was continued throughout the experiment. The mean value of IgG anti-BSA antibody in treated mice was 5·28 mg/ml and that in the control was 13·68 mg/ml, while the mean values of total serum IgG determined by radial immunodiffusion in these animals were 11·57 mg/ml and 16·33 mg/ml, respectively. This suggests that the suppression of IgG antibody production by Pro-boroPro is selective for anti-BSA antibody. In each experiment, we also measured IgM anti-BSA antibody and observed similar suppression by DP IV inhibitors to that of IgG. No abnormality was observed in the general appearance, behaviour, body weight and urinary protein in either the control or treated mice during these experiments.

DISCUSSION

In this study, we tested the synthetic DP IV inhibitors Ala-boroPro and Pro-boroPro *in vivo*, and found that both compounds, administered subcutaneously, inhibit DP IV in serum and spleen cell suspensions, and suppress antibody production in mice immunized with BSA with or without adjuvant. Pro-boroPro has been shown previously to suppress proliferation and IL-2 production of antigen stimulated $CD4^+$ murine T cell line *in vitro* [11]. The concentration of Pro-boroPro needed to effect 50% inhibition of proliferation and IL-2 production was about 5×10^{-7} M. In contrast, concanavalin A (Con A)-induced proliferation and IL-2 production of the same T cell line were

not inhibited even at as high concentration of inhibitor as 10^{-4} M. This indicated that blocking of the enzyme activity of DP IV by Pro-boroPro abrogates antigen-induced immune response selectively.

Subcutaneous injection of 20 μg of Pro-boroPro inhibited more than 90% of DP IV activity in mouse plasma. It is difficult to estimate the actual concentration of the inhibitor in blood, but if the inhibitor is absorbed immediately, the maximal concentration may reach about 10^{-6} M after injection of 20 μg. Since 10^{-7} M of Ala-boroPro or Pro-boroPro inhibits 90% of serum/plasma DP IV activity *in vitro* (data not shown), and half lives of free Ala-boroPro and Pro-boroPro are as short as 2–30 min and 1·5 h, respectively [11], the amount of inhibition *in vivo* by the injected doses is consistent with the *in vitro* results. When we increased the dosage of Pro-boroPro up to 160 μg, plasma DP IV was almost completely inhibited, and importantly, no sign of toxicity was observed in the mice.

Frequent administration of the inhibitors was required to maintain the serum DP IV activity at a low level. When we increased the dosage of the inhibitors up to 100 μg, even after consecutive injections, about 50% of normal DP IV activity was observed 23 h after the last injection. A spleen cell suspension always had a slightly higher DP IV activity than the corresponding plasma sample from the same inhibitor-treated mouse. One possible explanation for this difference between plasma and cellular DP IV might be new synthesis and release of enzyme by living cells during the preparation of cell suspensions. However, this does not appear to be a major factor, because cell-free serum DP IV activity recovered *in vitro* at the same rate as did enzyme activity *in vivo* (data not shown). Although Ala-boroPro and Pro-boroPro follow the slow-binding inhibition mechanism and produce quite stable enzyme-inhibitor complexes [11], these results suggested that the recovery of the inhibited DP IV activity is mainly due to dissociation and inactivation of the inhibitors. Since it took about 2 h to perform the cellular DP IV assay, the inhibition of cellular DP IV activity in the spleen may be more complete just after injection than our data indicate. We estimate that DP IV activity in serum and spleen can be kept at a level lower than 30% of normal by injections of more than 10 μg of Ala-boroPro or Pro-boroPro at 12 h intervals.

Changes in serum DP IV activity following the immunization of mice offer evidence that this enzyme is involved in *in vivo* immune responses. According to our data, serum DP IV activity 12–26 days after immunization with BSA was significantly higher than the pre-immunization level. This result may reflect the increased number of DP IV$^+$ T cells and density of DP IV per cell as is the case with T cells stimulated with mitogens *in vitro* [16]. We cannot explain why serum DP IV levels decrease while cell surface activity increases 2 days after immunization. Since we confirmed that mild anaemia by bleeding does not affect DP IV activity, this transient drop of serum DP IV may be caused by an acute increase of lymphokines which may be physiological substrates of DP IV, or decreased shedding of DP IV from T cells by binding of cellular DP IV to the natural ligand expressed on interacting cells.

Administration of Ala-boroPro and Pro-boroPro caused only partial suppression of anti-BSA antibody production in these experiments. The residual antibody levels may reflect the fact that about 10–30% of DP IV activity remained during the treatment period. We started the injections of DP IV inhibitors 1 h after (experiment 1) or 1 h before (experiments 2–4) immunization; either way, a fraction of T cells might have escaped and started a series of intracellular reactions leading to proliferation, cytokine production, and cytokine-receptor expression. It is not known even in *in vitro* studies whether DP IV inhibitors suppress the function of T cells which have already been activated.

Recent *in vitro* studies have provided clues to the mechanism of suppression of antibody production by DP IV inhibitors. First, inhibitors of DP IV may interfere with the processing of some lymphokines which have an essential role in the proliferation of helper T cells and B cells. Since some of the lymphokines such as IL-1β and IL-2 have a susceptible site for DP IV activity, it is possible that interaction with DP IV is necessary to exert maximal activities for these lymphokines. We have observed that human recombinant IL-2 can be cleaved by purified DP IV; how this may affect lymphokine activity is under investigation.

A second hypothesis is that DP IV may work synergistically with the CD3/TCR pathway as an important accessory molecule in T cell activation. Fleischer [17] reported that cross-linking of the T cell activation molecule Tp103 via a MoAb leads to an IL-2-dependent proliferative response of CD3$^+$ T lymphocytes. Recently, an affinity purified Tp103 fraction has been shown to be associated with DP IV activity [18]. In addition, the slow turnover of Tp103 on activated T cells stimulated by mitogen or in mixed lymphocyte culture (MLC) [17] is consistent with our observation of *in vivo* DP IV activity following immunization. Another line of *in vitro* studies supporting this hypothesis comes from studies with a MoAb, anti-1F7 described by Morimoto and colleagues [19–21]. Soluble anti-1F7 inhibits antigen-driven T cell proliferation and helper function for pokeweed mitogen (PWM)-driven IgG synthesis [19], and immobilized anti-1F7 has a co-mitogenic effect with anti-CD3 or anti-CD2 in CD4$^+$ T cell activation [20]. It seems likely that DP IV is one of the molecules in an assembly recognized by anti-1F7, because a prominent structure recognized by anti-1F7 is a 110-kD molecule also recognized by anti-Ta1 (CD26) [19] which binds to DP IV [22,23]. It is not known whether these MoAbs inhibit DP IV enzyme activity. Nevertheless, these *in vitro* studies and our enzymological studies agree in indicating that DP IV plays an important role in immune system function.

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