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Cross-talk between Vβ8$^+$ and γδ$^+$ T lymphocytes in contact sensitivity

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SUMMARY

We have previously reported that T lymphocytes proliferating *in vitro* to the hapten trinitrochlorobenzene (TNCB) exhibit a very restricted Vβ gene usage and response to TNCB is limited to T-cell receptors (TCR) composed of Vβ8.2 in combination with Vα3.2, Vα8 and Vα10. This paper investigates the role played by T lymphocytes expressing the Vβ8.2 gene segment in the contact sensitivity (CS) reaction to TNCB in the intact mouse and in its passive transfer into naive recipient mice. Mice injected with monoclonal antibodies to Vβ8 are unable to develop CS upon immunization with TNCB and 4-day TNCB-immune lymph node cells from mice that had been depleted *in vivo* or *in vitro* of Vβ8$^+$ T lymphocytes fail to transfer CS. However, when separated Vβ8$^+$ and Vβ8$^-$ cells were used for passive transfer, it was found that Vβ8$^+$ T lymphocytes failed to transfer CS when given alone to recipient mice and a Vβ8$^-$ population was absolutely required. Further analysis revealed that within the Vβ8$^-$ population, T lymphocytes expressing the γδ TCR were fundamental to allow transfer of the CS reaction. These γδ cells were found to be antigen non-specific, genetically unrestricted and to rearrange the Vγ3 gene segment. This indicates that transfer of the CS reaction requires cross-talk between Vβ8$^+$ and γδ$^+$ T lymphocytes, thus confirming our previous results obtained using TNCB-specific T-cell lines. Time–course experiments showed that Vβ8$^+$ lymphocytes taken 4–24 days after immunization with TNCB were able to proliferate and produce interleukin-2 (IL-2) in response to the specific antigen *in vitro*. Similar time–course experiments were then undertaken using the passive transfer of the CS reaction system. The results obtained confirm that TNCB-specific Vβ8$^+$ T lymphocytes are present in the lymph nodes of immunized mice from day 4 to day 24, and reveal that γδ$^+$ T lymphocytes are active for a very short period of time, i.e. days 4 and 5 after immunization. In fact, TNCB-specific Vβ8$^+$ cells are able to transfer CS when taken 4–24 days after immunization, providing the accompanying Vβ8$^-$ or γδ$^+$ T lymphocytes are obtained 4 days after immunization. In contrast, injection of Vβ8$^+$ T lymphocytes together with Vβ8$^-$ or γδ$^+$ T lymphocytes that had been taken 2 or 6 days after immunization, failed to transfer significant CS into recipient mice. Taken together, our results confirm that cross-talk between Vβ8$^+$ and γδ$^+$ T lymphocytes is necessary for full development of the CS reaction and may explain why the CS reaction in the intact mouse lasts up to 21 days after immunization while the ability of immune lymph node cells to transfer CS is limited to days 4 and 5 after immunization.

INTRODUCTION

Hapten-specific T lymphocytes play an instrumental role in chemical- or drug-induced allergic disorders. In studies on hapten-induced T-cell responses *in vivo* and *in vitro*, nitrobenzene derivatives, such as trinitrochlorobenzene (TNCB) have been major experimental reagents.[1] Unlike conventional protein antigens, in which complexes of major histocompatibility complex (MHC) molecules and processed antigenic peptides have been identified as ligands for the antigen-specific T-cell receptor (TCR),[2,3] the molecular basis for the recognition of haptens by T cells is not yet well understood.[4] Chemically reactive haptens bind covalently to proteins, in general on the surface of cells. The lack of selectivity in these reactions leads to a multitude of individual hapten epitopes. Moreover, hapten determinants may be covalently attached to MHC molecules or to MHC-associated peptides or to proteins that are subsequently processed. However, recent work suggests that there may be TNCB epitopes recognized by the TCR,[5,6] indicating that recognition of TNCB occurs via mechanisms similar to those leading to recognition of conventional peptide antigens.

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This leads to significant skewing in selection of the TCR repertoire in response to TNCB. In fact, we and others have reported that T lymphocytes proliferating *in vitro* to TNCB exhibit a very restricted Vβ gene usage and $CD4^+$ T-cell response to TNCB seems to be limited to TCR composed by Vβ8.2[7] in combination with Vα3.2, Vα8[6] and Vα10.[8]

Analysis of the role played by $V\beta8^+$ T lymphocytes during *in vivo* responses to TNCB, have been undertaken using TNCB-specific T-cell lines. These lines, which are 90–94% $V\beta8.2^+$ and 6–9% $\gamma\delta^+$, fail to mediate systemic passive transfer of the contact sensitivity (CS) reaction, unless the recipient mice or the cell lines are exposed to low doses interleukin-4 (IL-4).[9] Analysis showed that both the $V\beta8^+$ and the $\gamma\delta^+$ cells were absolutely required to transfer CS and that IL-4 acts on the $\gamma\delta^+$ T lymphocytes which express IL-4 receptor (IL-4R) and bind it. However, the possibility remains that the results obtained using TNCB-specific T-cell lines might have been due to an artifact derived from the prolonged *in vitro* culture with antigen and IL-2, generating a skewed population, and therefore they might not be representative of a truly *in vivo* response.

Against this background, we decided to investigate the role played by $V\beta8^+$ T lymphocytes in two examples of immune response to TNCB *in vivo,* namely the CS reaction and its passive transfer.

MATERIALS AND METHODS

Mice

Male mice of the strains CBA/J and BALB/c were obtained from OLAC Ltd. (Bicester, UK) and bred at the Institute of General Pathology, under facility conditions. CBA/J mice were used in all the experiments, while BALB/c mice were only used in the genetic restriction experiments reported in Fig. 4. Eight-to 12-week-old mice were used in each experiment and each experimental group consisted of five to eight mice.

Contact sensitivity

Mice were immunized epicutaneously with 0·1 ml 5% TNCB (BDH, Poole, UK) or 3% oxazolone (OX; BDH) dissolved in acetone:ethanol (1:3) and applied to the shaved skin of the thorax and abdomen. Four to five days later, the mice were challenged by applying 20 μl 0·1% TNCB in olive oil on both sides of both ears. The increase in ear thickness was measured 24 hr later with an engineer's micrometer and was expressed in units of 10^{-3} cm ± standard deviation (SD).

Passive transfer of contact sensitivity

Mice were immunized with TNCB as described above and the draining lymph node cells were harvested 4 days later. The cells were washed three times in medium RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (Life Technologies), penicillin, streptomycin, glutamine and 2-mercaptoethanol (2-ME; 2×10^{-5} M) and 5×10^7 cells in 0·5 ml were injected intravenously (i.v.) into naive recipient mice. The mice were challenged immediately with TNCB and increase in ear thickness was measured after 24 hr.

Preparation of antigen-presenting cells

TNCB-modified spleen cells were used as antigen *in vitro* and prepared as previously described.[10] Briefly, normal spleen cells were depleted of red cells by Boyle's solution (3 min, room temperature). The cells were diluted with medium, spun down, washed three times and irradiated (30 Gy, from a caesium source). After three more washings, the cells [5×10^7/ml in phosphate-buffered saline (PBS)] were treated with 5 mM 2,4,6-trinitrobenzenesulphonic acid (TNPSA; BDH) which had been brought to pH 7·2 with sodium bicarbonate. After 10 min at room temperature, the cells were washed once in PBS and three more times in medium.

Proliferation assay

Four-day TNCB-immune lymph node cells (2×10^5 in 100 μl) were cultured at 37° in the presence of 5% CO_2 in flat-bottomed 96-well microtitre plates (Nunc, Copanhagen, Denmark) with TNCB-modified syngeneic spleen cells, as antigen-presenting cells (APC, 4×10^5 in 100 μl).[11,12] Three days later, the wells were pulsed with 1 μCi methyl-[^{3}H]thymidine (Amersham, Bucks, UK) and harvested 18 hr later. Results are expressed as mean counts per minute of triplicate wells ± SD. SD were usually less than 10% of the means.

IL-2 production and assay

Four-day TNCB-immune lymph node cells were cultured with TNCB-APC as described in the above section.[11,12] After 24 hr, supernatants were collected by centrifugation, filtered and stored at −70°. IL-2 activity of supernatants was measured by their ability to support the growth of cytotoxic T lymphocytes (CTLL). Briefly, CTLL (5×10^4 in 50 μl) were added to 50 μl of serial dilutions of supernatants in flat-bottomed microwell plates (Nunc) and incubated at 24 hr. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, 5 mg/ml, 20 μl per well] was added and the reaction was stopped after 4 hr with 100 μl 10% sodium dodecyl sulphate (SDS). After overnight incubation, the absorbance (570–630 nm) was measured with an enzyme-linked immunosorbent assay (ELISA) reader.[13] Because IL-4 also allows survival of CTLL, the supernatants were absorbed twice in microtray wells coated with the anti-IL-4 monoclonal antibody (mAb) 11B11, according to our published method. IL-2 activity of supernatants is expressed in units (U)/ml, i.e. the reciprocal of the dilution giving 50% maximal response.

Preparation of T-cell subsets by immunomagnetic bead separation

TNCB-immune lymph node cells were incubated with a mixture of fluorescein-conjugated anti-B220 and anti-I-A^k mAb for 30 min at 4°. The cells were then washed twice and resuspended with sheep anti-fluorescein isothiocyanate-coated magnetic beads (Advanced Magnetics, Cambridge, MA) at a 3:1 ratio. The mixture of cells and beads was kept in a flat-walled flask in an ice bath. After 15 min, cells that had bound antibody were depleted by two rounds of exposure to a magnetic field. The residual (non-adherent) cells were removed, washed three times and used as negatively selected enriched T cells (95% $CD3^+$ T cells). This non-adherent population was then incubated with anti-Vβ8 TCR mAb (2 μg/10^6 cells, F23.1, a gift of Prof. K. Tomonari, Department of Immunology and Parasitology, Fukui Medical School, Japan),[14] followed by anti-mouse immunoglobulin-coated beads (Biomag, 1 mM iron magnetic particles, Advanced Magnetics, Cambridge, MA).

The adherent cells were recovered and used as a source of Vβ8$^+$ T lymphocytes, while non-adherent cells were used as a source of Vβ8$^-$ T lymphocytes. For further cell separation, these Vβ8$^-$ cells were incubated with anti-αβ (H57-597, PharMingen, San Diego, CA) or anti-γδ TCR mAb (GL3, PharMingen), at 2 μg/10^6 cells, and then with anti-hamster immunoglobulin-coated magnetic beads. When the Vγ region gene usage of the γδ cells was studied, the Vβ8$^-$ cells were incubated with mAb anti-Vγ1 (a kind gift of Prof. S. Tonegawa, MIT, Bethesda, MD and Dr P. Pereira, Institut Pasteur, Paris, France),[15] anti-Vγ2 (PharMingen) or anti-Vγ3 (PharMingen) and then with anti-hamster immunoglobulin-coated magnetic beads. The adherent cells were recovered and used as a source of γδ$^+$ T lymphocytes. Using these procedures, the purity of the adherent population was always over 90% and their viability exceeded 95%, as assessed by fluorescence-activated cell sorter (FACS) analysis.

For transfer experiments, the mixtures of cells and beads were injected intraperitoneally (i.p.). In these experiments the mice were challenged on the ears 24 hr later and increase in ear thickness was measured after a further 24 hr.

Treatment of lymph node cells with antibody and complement

Four-day TNCB-immune lymph node cells (10^8/ml) were incubated for 1 hr at 4° with the anti-Vβ8 (F23.1) or the anti-Vβ17a (KJ23, a gift of Prof. K. Tomonari) mAb at a final concentration of 10 μg/ml. After washing and centrifugation, the cells were incubated for 1 hr at 37° with low-tox rabbit complement (Cedarlane, Ontario, Canada) diluted 1/20.

In vivo *injection of mAb*

Mice were injected i.p. with a total of 1 mg of the anti-Vβ8 mAb F23.1.[7] The mice received a second i.p. injection (500 μg) 48 hr later and were immunized with TNCB 72 hr after the first injection of the mAb. As a control, mice were injected i.p. with the isotype-matched mAb KJ23 (anti-Vβ17a) using the same protocol as F23.1.[7] Depletion of Vβ8$^+$ cells was assessed by FACS analysis as described above.

Statistics

The double-tailed Student's *t*-test was used to analyse the significance of the differences between groups.

RESULTS

Depletion of Vβ8$^+$ cells abrogated CS reaction to TNCB and its passive transfer

Previous results from our laboratories showed that Vβ8$^+$ T lymphocytes may play an important role in certain T-cell responses to TNCB *in vitro*.[7] Here we explore the relevance of Vβ8$^+$ cells in the CS reaction to TNCB *in vivo*. Mice were depleted of Vβ8$^+$ cells by injection af the anti-Vβ8 mAb F23.1 starting 3 days before epicutaneous immunization with TNCB. Depletion of Vβ8$^+$ cells from lymph nodes was 99–99·7% complete at the time of immunization of the mice with TNCB (data not shown). Figure 1 shows that injection of mice with F23.1 mAb caused a strong inhibition of the CS reaction, while injection of the isotype-matched control mAb KJ23 (anti-Vβ17a) had virtually no effect on the CS reaction. Similar results were obtained in two additional experiments. This suggests that Vβ8$^+$ lymphocytes are critical in the CS reaction to TNCB.

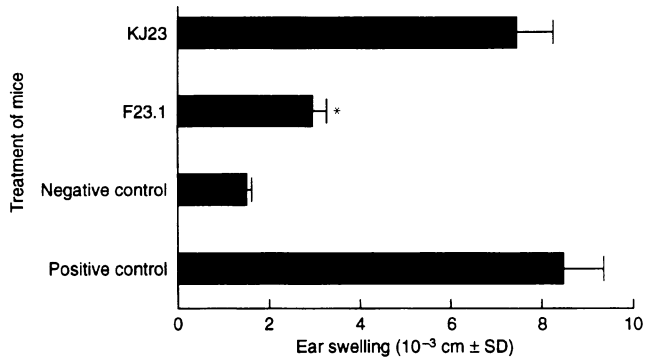

Figure 1. Contact sensitivity reaction to TNCB in mice acutely depleted of Vβ8$^+$ cells by mAb *in vivo* before immunization. CBA J mice were injected i.p. with 1 mg of the anti-Vβ8 mAb F23.1 The mice received a second i.p. injection (500 μg) 48 hr later and were immunized with TNCB 72 hr after the first injection of the mAb. As a control, mice were injected i.p. with the isotype-matched mAb KJ23 (anti-Vβ17a). The positive control consists of mice that were immunized and challenged with TNCB but were not treated with mAb. The negative control consists of mice that were not immunized but were only challenged with TNCB. *$P<0.005$ when compared to the positive control group.

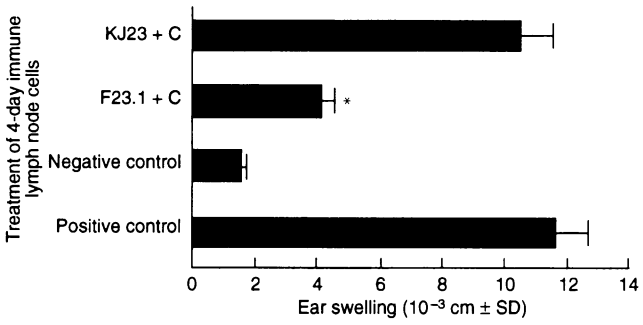

Figure 2. Removal of Vβ8$^+$ cells from 4-day TNCB-immune lymph node cells abolishes the passive transfer of contact sensitivity. Four-day TNCB-immune lymph node cells were treated with mAb and complement and the equivalent of 5×10^7 cells in 0·5 ml were injected i.v. into naive recipient mice. The mice were challenged immediately with TNCB and increase in ear thickness was measured after 24 hr. *$P<0.001$ when compared to the positive control group.

To confirm the requirement for Vβ8$^+$ lymphocytes in the CS reaction to TNCB, passive transfer experiments were undertaken. Mice were immunized with TNCB, lymph node cells were harvested 4 days later and were treated *in vitro* with the anti-Vβ8 mAb F23.1 and complement. Figure 2 shows pooled results from two different experiments. Depletion of Vβ8$^+$ cells from the 4-day TNCB-immune lymph node population caused a failure in the capacity of such cells to transfer the CS reaction into recipient mice. Treatment of the 4-day TNCB-immune lymph node population with the control mAb KJ23 had no effect on the ability to transfer the CS reaction. It may be concluded that Vβ8$^+$ lymphocytes play a pivotal role in the CS reaction to TNCB.

Vβ8$^+$ lymphocytes are necessary, but not sufficient, to allow passive transfer of the CS reaction

We have previously reported that purified Vβ8$^+$ TNCB-immune lymphocytes from BALB/c mice fail to transfer systemically the CS reaction into naive recipient mice.[16] Furthermore, using TNCB-specific T-cell lines, we have reported that both Vβ8.2$^+$ and γδ$^+$ T lymphocytes are important in the systemic passive transfer of the CS reaction to TNCB.[9] We therefore asked whether, in the passive transfer of the CS reaction carried out by bulk 4-day TNCB-immune lymph node cells, Vβ8$^+$ lymphocytes alone were able to transfer the CS reaction, or whether other cells were required. For this purpose, mice were immunized with TNCB and lymph node cells were harvested 4 days later. Vβ8$^+$ and Vβ8$^-$ cells were obtained by immunomagnetic bead separation and were transferred into naive recipient mice.

Figure 3a shows that both Vβ8$^+$ and Vβ8$^-$ cells failed to transfer CS systemically when injected alone, but both cells together were fully active. Similar results were obtained in one additional experiment. This observation indicates that Vβ8$^+$ cells are important in the passive transfer of the CS reaction but other Vβ8$^-$ cells are also required.

To ascertain which cell within the Vβ8$^-$ population was important in the passive transfer of the CS reaction, the Vβ8$^-$ population was further depleted of αβ$^+$ or γδ$^+$ cells and cotransferred together with Vβ8$^+$ cells into naive recipient mice. Figure 3b shows that injection of Vβ8$^+$ and Vβ8$^-$ cells transferred the CS reaction, and removal of αβ$^+$ cells from the Vβ8$^-$ population did not modify the ability of this population to allow transfer of CS by Vβ8$^+$ cells. Removal of γδ$^+$ cells from the Vβ8$^-$ population abolished the passive transfer of CS. This requirement for γδ$^+$ cells was confirmed by the results showing that injection of Vβ8$^+$ cells and γδ$^+$ cells (isolated from the Vβ8$^-$ population) was fully able to transfer the CS reaction. Two additional experiments gave similar results. From these results we conclude that an efficient transfer of the CS reaction to TNCB requires cross-talk between Vβ8$^+$ and γδ$^+$ T lymphocytes.

γδ$^+$ cells which allow Vβ8$^+$ cells to transfer CS are antigen non-specific, genetically unrestricted and rearrange the Vγ3 gene

Vβ8$^+$ T cells from TNCB-immune lymph nodes were injected into recipient mice together with γδ$^+$ T lymphocytes taken from TNCB- or OX-immune mice, or from normal (i.e. unimmunized) mice, and CS reaction was assessed. Figure 4a shows that γδ$^+$ T lymphocytes obtained from both TNCB- or OX-immune mice were able to allow transfer of CS by Vβ8$^+$ T lymphocytes, thus indicating the antigen-non-specific nature of these cells. However, prior sensitization was required, as γδ$^+$ T lymphocytes taken from unimmunized mice failed to allow transfer of CS by Vβ8$^+$ T lymphocytes. In fact, injection of mice with γδ$^+$ T lymphocytes from the lymph nodes of non-immune mice together with TNCB- or OX-immune Vβ8$^+$ T lymphocytes gave an ear swelling increase of 2·2±1·5 and 2·0±0·7 units, respectively. Furthermore, the activity of γδ$^+$ T lymphocytes was also genetically unrestricted as

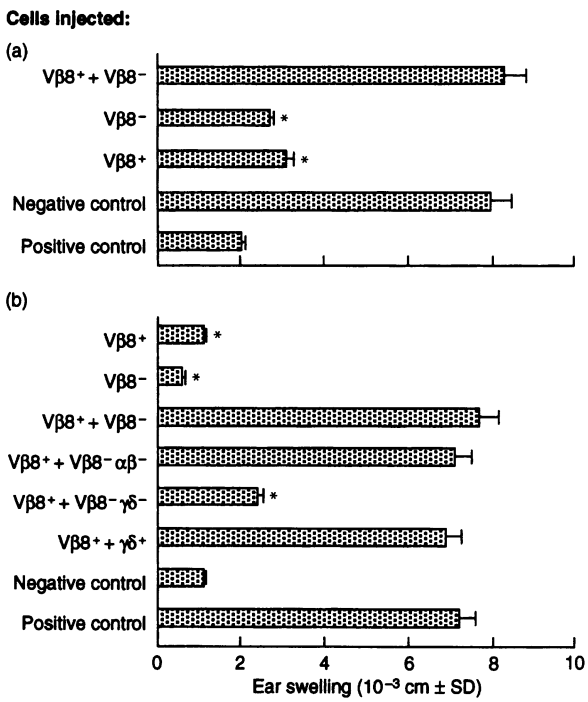

Figure 3. Transfer of contact sensitivity to TNCB requires the participation of Vβ8$^+$ and Vβ8$^-$ γδ$^+$ cells. Vβ8$^+$ and Vβ8$^-$ T cells were separated from 4-day TNCB-immune lymph node cells by immunomagnetic beads. The mixtures of cells and beads were injected i.p. In these experiments, the mice were challenged on the ears 24 hr later and increase in ear thickness was measured after a further 24 hr. *$P<0·005$ when compared to the positive control group.

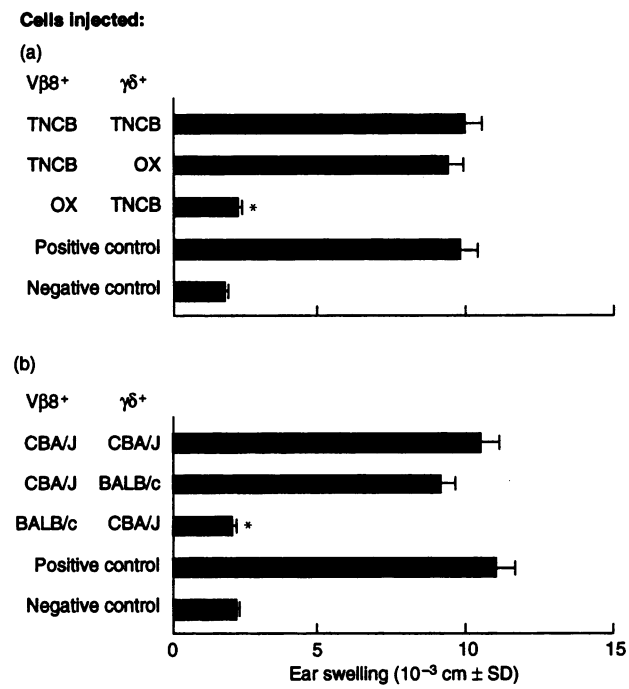

Figure 4. Antigen-specificity and genetic restriction of the γδ$^+$ T lymphocytes involved in the transfer of the CS reaction. (a) Mice were injected i.p. with Vβ8$^+$ and γδ$^+$ T lymphocytes sorted from the lymph nodes of day 4 TNCB- or OX-immune mice. (b) Mice were injected i.p. with Vβ8$^+$ and γδ$^+$ T lymphocytes sorted from the lymph nodes of day 4 TNCB-immune CBA/J or BALB/c mice. *$P<0·005$ when compared to the positive control group.

demonstrated by the ability of $\gamma\delta^+$ T lymphocytes from TNCB-immune BALB/c mice to allow transfer of CS by $V\beta8^+$ T lymphocytes from TNCB-immune CBA/J mice (Fig. 4b). Two additional experiments confirmed the antigen-non-specific and genetically unrestricted nature of the $\gamma\delta^+$ T lymphocytes.

The Vγ gene usage of the $\gamma\delta^+$ T lymphocytes involved in the passive transfer of CS was investigated in the following experiment. Briefly, $V\beta8^+$ T cells were injected into recipient mice together with $V\beta8^-$ cells from which $V\gamma1^+$, $V\gamma2^+$, or $V\gamma3^+$ γδ T lymphocytes were removed by immunomagnetic beads. Figure 5 shows that removal of $V\gamma1^+$ or $V\gamma2^+$ T lymphocytes from the $V\beta8^-$ population had no effect on the ability of these cells to allow transfer of CS, while removal of $V\gamma3^+$ T lymphocytes abolished the ability of the $V\beta8^-$ population to allow transfer of the CS. These results, together with similar ones obtained in two additional experiments, clearly demonstrate that the $\gamma\delta^+$ T lymphocytes involved in the transfer of CS rearrange the Vγ3 gene region.

Time–course of the appearance and action of $V\beta8^+$ and $\gamma\delta^+$ T lymphocytes

There is a dissociation between the CS reaction in the intact mouse and its passive transfer into naive recipient mice. In fact, following epicutaneous immunization with TNCB, the CS reaction in the intact mouse is measurable 4–21 days later,[17] while the passive transfer into naive recipient mice by immune lymph node cells is virtually confined to days 4 and 5 after immunization.[18] The findings that both $V\beta8^+$ and $\gamma\delta^+$ lymphocytes are required to transfer the CS reaction to TNCB, raised the question of whether the time–course of the ability of lymph node cells to transfer the CS reaction was determined by the time–course of $V\beta8^+$ or $\gamma\delta^+$ T lymphocytes.

To assess this issue we first analysed the proliferative and IL-2 responses to TNCB *in vitro* displayed by $V\beta8^+$ and $V\beta8^-$ T lymphocytes taken from mice at various times after epicutaneous immunization with TNCB. Figure 6 shows that $V\beta8^-$ lymphocytes virtually failed to proliferate (Fig. 6a) and produce IL-2 (Fig. 6b) upon stimulation with TNCB *in vitro*, irrespective of the time at which the lymph node cells were harvested. As a control, $V\beta8^-$ lymphocytes were able to give good proliferative and IL-2 responses upon stimulation with Concanavalin A (data not shown). When the response of $V\beta8^+$ lymphocytes was studied, it was found that $V\beta8^+$ lymphocytes taken from mice 2 days after immunization with TNCB, failed to proliferate and produce IL-2 in response to TNCB *in vitro*, but the same cell population taken 4–24 days after immunization, gave strong antigen-specific proliferative and IL-2 responses.

We therefore carried out passive transfer experiments using $V\beta8^+$ and $V\beta8^-$ lymphocytes taken from donor mice at various times after epicutaneous immunization with TNCB. Figure 7 shows that $V\beta8^+$ lymphocytes taken from mice 2 days after immunization failed to transfer the CS reaction, irrespective of the time at which $V\beta8^-$ lymphocytes were harvested (i.e. 2, 4, or 6 days after immunization). $V\beta8^+$ lymphocytes taken 4–24 days after epicutaneous immunization with TNCB were fully able to transfer CS providing $V\beta8^-$ lymphocytes taken 4 days after immunization were co-transferred. In contrast, $V\beta8^-$ lymphocytes taken 2 or 6 days after immunization failed to co-operate with $V\beta8^+$ lymphocytes in the passive transfer of CS.

To identify which cell within the $V\beta8^-$ population was responsible for the transfer of the CS reaction, $V\beta8^-$ lymphocytes were taken at 2, 4 and 6 days after immunization and $\gamma\delta^+$ lymphocytes were isolated by immunomagnetic bead separation. The $\gamma\delta^+$ cells so obtained were co-injected with $V\beta8^+$ lymphocytes taken 4–24 days after immunization. Figure 8 shows that $\gamma\delta^+$ cells were able to allow systemic transfer of CS by $V\beta8^+$ cells, when taken 4 days after immunization, but not when taken 2 or 6 days after immunization. From these results we conclude that $V\beta8^+$ cells are present in the lymph nodes of TNCB-immune mice for a long period of time after immunization with TNCB, while $\gamma\delta^+$ cells are only present for a very short period of time (i.e. 4 days after immunization). Furthermore, the time–course of the passive transfer of the CS reaction is strictly correlated to the time–course of the $\gamma\delta^+$ cells. Three additional experiments confirmed the time–course results reported in Figs 6, 7 and 8.

DISCUSSION

This study was prompted by the observation that T lymphocytes responding *in vitro* to the hapten TNCB show a dominant

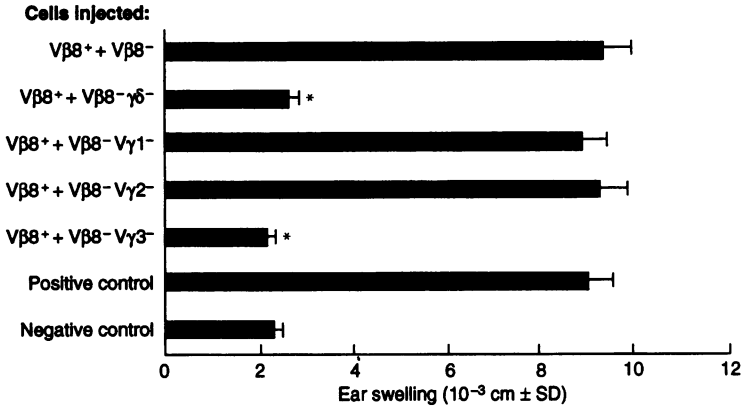

Figure 5. $\gamma\delta^+$ T lymphocytes involved in the transfer of the CS reaction rearrange the Vγ3 gene region. $V\beta8^+$ and $V\beta8^-$ T cells were separated from 4-day TNCB-immune lymph node cells by immunomagnetic beads. The $V\beta8^-$ cells were incubated with mAb anti-Vγ1, anti-Vγ2 or anti-Vγ3 and then with anti-hamster immunoglobulin-coated magnetic beads. The adherent cells were recovered and used as a source of $\gamma\delta^+$ T lymphocytes. $^*P<0{\cdot}001$ when compared to the positive control group.

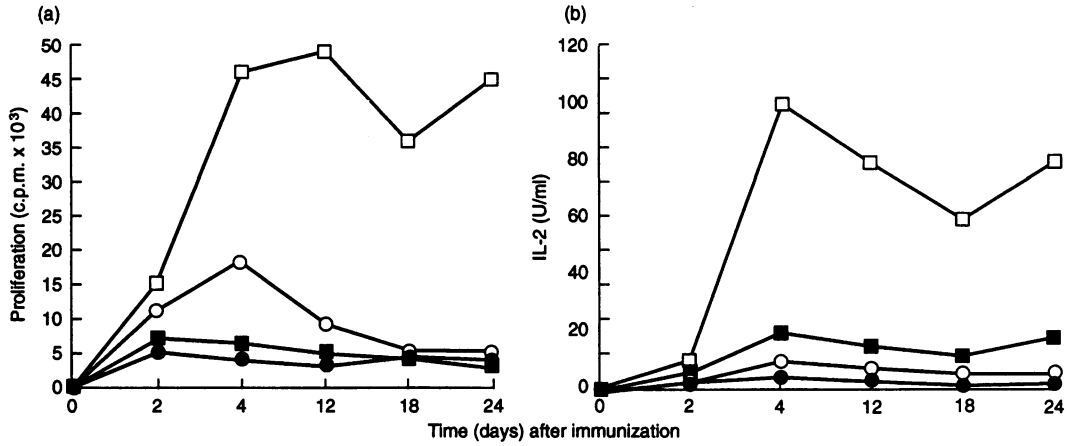

Figure 6. Time-course of the antigen-specific proliferative response and IL-2 production by TNCB-immune Vβ8$^+$ and Vβ8$^-$ cells. Lymph node cells were taken at different times after immunization with TNCB. They were separated into Vβ8$^+$ (squares) and Vβ8$^-$ (circles) cells and re-exposed to the specific antigen (open symbols) or medium (filled symbols) *in vitro*. The cells were then tested for their ability to proliferate (a) and to produce IL-2 (b). In this experiments, equal numbers of Vβ8$^+$ and Vβ8$^-$ cells were used.

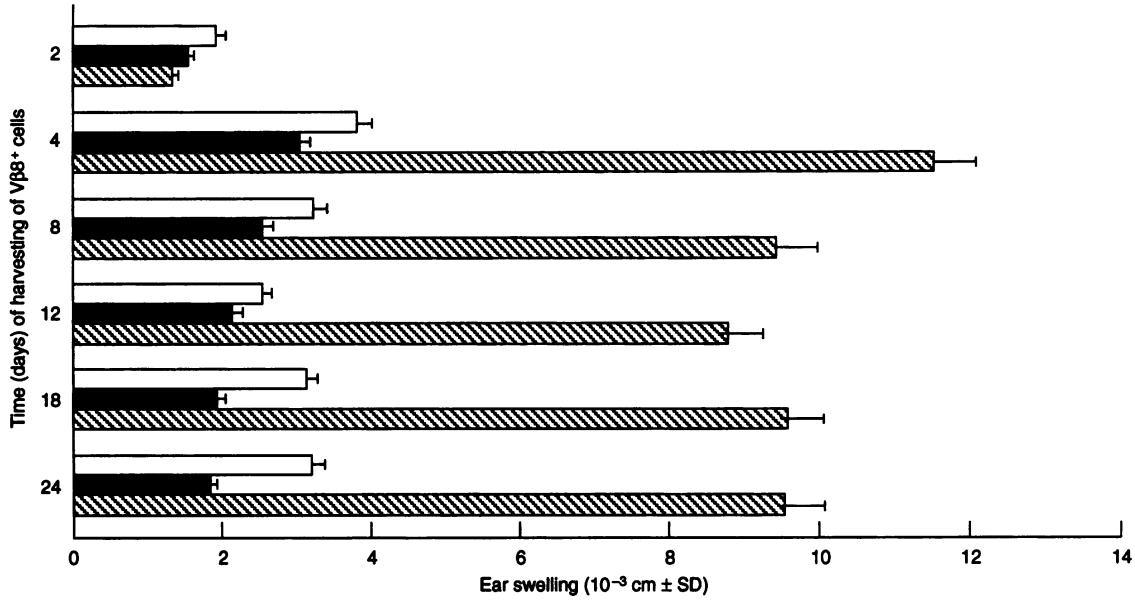

Figure 7. Time-course of the ability of Vβ8$^+$ and Vβ8$^-$ cells to transfer contact sensitivity. Naive recipient mice were injected with Vβ8$^+$ cells taken at various times after immunization with TNCB and Vβ8$^-$ cells taken at 2 (open blocks), 4 (hatched blocks) and 6 (filled blocks) days after immunization with TNCB. The mice were immediately challenged and tested for contact sensitivity. Negative and positive control values were $1{\cdot}5 \pm 0{\cdot}5$ and $9{\cdot}8 \pm 0{\cdot}7$, respectively.

Vβ8 gene usage and their removal *in vivo* or *in vitro* abolishes the proliferative response of lymph node cells taken from mice immunized epicutaneously with TNCB.[7] The aim of this paper was therefore to investigate the role played by cells rearranging the Vβ8 gene segment in the CS reaction to TNCB.

The present results confirm the importance of Vβ8$^+$ cells in the CS reaction to TNCB and show that *in vivo* injection of the anti-Vβ8 mAb F23.1 before immunization abolishes the development of the CS reaction in the intact mouse. Furthermore, *in vitro* depletion of Vβ8$^+$ cells abolished the ability of lymph node cells taken 4 days after immunization with TNCB to transfer systemically the CS reaction. However, experiments reported in Fig. 3 clearly show that Vβ8$^+$ cells, although necessary, were not sufficient for a successful systemic passive transfer of the CS reaction, and a Vβ8$^-$ cell population was absolutely required. A deeper analysis revealed that within the Vβ8$^-$ population, γδ$^+$ cells were the key cells in allowing a systemic transfer of the CS reaction.

Our results are in agreement with the observation of Ptak and Askenase[19] who reported that the systemic transfer of CS requires both αβ and γδ T lymphocytes. Furthermore, these results confirm our previous studies on antigen (TNCB)-specific T-cell lines. These lines, which are 90–94% Vβ8.2$^+$ and 6–9% γδ$^+$, mediate systemic passive transfer of the CS reaction, providing the recipient mice or the cell lines are exposed to low doses of IL-4. Analysis showed that IL-4 acts

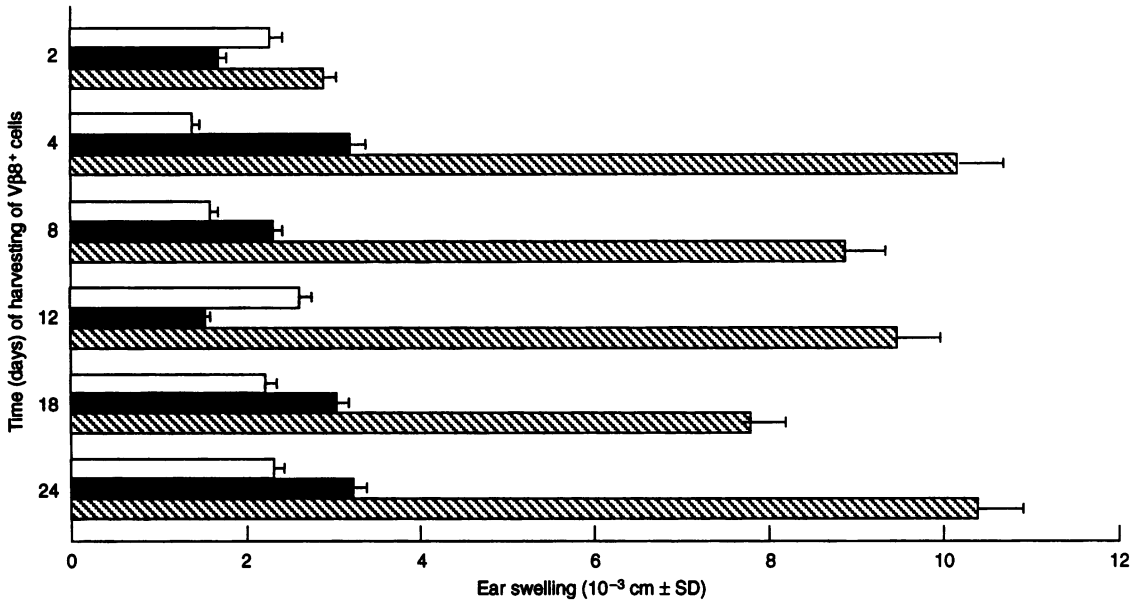

Figure 8. Time–course of the ability of Vβ8$^+$ and γδ$^+$ cells to transfer contact sensitivity. Naive recipient mice were injected with Vβ8$^+$ cells taken at various times after immunization with TNCB and γδ$^+$ cells taken at 2 (open blocks), 4 (hatched blocks) and 6 (filled blocks) days after immunization with TNCB. The mice were immediately challenged and tested for contact sensitivity. Negative and positive control values were $1{\cdot}8 \pm 0{\cdot}6$ and $10{\cdot}4 \pm 1{\cdot}2$, respectively.

on the γδ$^+$ T lymphocytes which express IL-4R and bind it.[9] Finally, the findings reported in this paper, showing that both Vβ8$^+$ and γδ$^+$ T lymphocytes are required for the systemic transfer of CS carried out by bulk 4-day TNCB-immune lymph node cells, exclude the possibility that the results obtained using TNCB-specific T-cell lines[9] might have been due to an artifact derived from the prolonged *in vitro* culture with antigen and IL-2, generating a skewed population.

In general, the CS reaction in the intact mouse lasts up to 21 days after immunization.[17] Similarly, Vβ8$^+$ cells are present in the lymph nodes of TNCB-immune mice up to 24 days after immunization, at least when tested for their ability to proliferate and produce IL-2 upon exposure to the specific antigen *in vitro* (Fig. 5). However, the ability of lymph node cells to transfer CS systemically is virtually limited to days 4 and 5 after immunization.[18] The question then arises: is the time–course of the transfer of the CS reaction dictated by the time–course of the Vβ8$^+$ or γδ$^+$ T lymphocytes? For this purpose, Vβ8$^+$ lymphocytes were taken at various times after immunization with TNCB and were cotransferred into recipient mice together with Vβ8$^-$ or with positively sorted γδ$^+$ T lymphocytes taken 2, 4, or 6 days after immunization. The key findings were:

(1) Vβ8$^+$ T lymphocytes taken 2 days after immunization fail to transfer CS, irrespective of the time at which Vβ8$^-$ or γδ$^+$ T lymphocytes were taken;

(2) Vβ8$^+$ T lymphocytes taken 4–24 days are fully able to transfer the CS reaction providing Vβ8$^-$ or γδ$^+$ T lymphocytes obtained 4 days after immunization are present;

(3) Vβ8$^-$ or γδ$^+$ T lymphocytes are able to allow systemic passive transfer of the CS reaction, carried out by Vβ8$^+$ T lymphocytes, only when taken 4 days after immunization, but not when taken 2 or 6 days after sensitization.

Why are γδ$^+$ T lymphocytes only detectable for a very limited period of time (4 days after immunization) by transfer experiment, although the CS reaction in the intact animal persists for at least up to 3 weeks after immunization? It is likely that before day 4 γδ$^+$ T lymphocytes cannot be detected because they are too few in number. After day 4–5, they may be absent or inactive because of down-regulation by certain *suppressor* cell populations, or cytokines, or because this is their intrinsic natural history. Alternatively, after day 4–5 γδ$^+$ T lymphocytes may exist but escape detection because they need restimulation by antigen. These possibilities are not mutually exclusive, as suggested by studies in mice transgenic for a γδ T-cell receptor specific for the product of the T22 gene of the *Tla* locus.[20] In fact, after *in vivo* immunization with the specific alloantigen, γδ$^+$ T lymphocytes in the spleen proliferate reaching a peak at days 4 and 5 and subsequently many of them undergo programmed cell death (apoptosis).

However, besides a similar time–course, our system has some important differences with the transgenic one. In fact, expansion of γδ cells following immunization with TNCB does not seem to be due to proliferation of cells in response to antigen *in situ* in the lymph nodes. Three main observations support this assumption: first, expansion of γδ cells in the lymph nodes of mice immunized with TNCB is not affected by *in vivo* treatment with hydroxyurea (FD and GS, unpublished observations); second, the vast majority of γδ cells expanding in the lymph nodes following immunization with TNCB and accumulating at the site of antigen challenge during CS rearrange the Vγ3 gene segment[21] which is the same gene segment used by dendritic epidermal T cell (DETC); third, the γδ cells involved in the passive transfer of the CS reaction similarly rearrange the Vγ3 gene reaction (Fig. 6). Therefore, it is conceivable to suppose that the γδ cells expanding in the lymph nodes after immunization with TNCB might come directly from the skin.[21,22]

Although we have not evaluated possible apoptosis of γδ cells in the lymph nodes, we favour the possibility that these

cells cannot be detected by 4 days after immunization because they migrate elsewhere. In fact, Sprent and Miller[23] suggested that activated T cells migrate to the gut, where they are excreted or die. This possibility might explain the long-lasting CS reaction in the intact mouse with respect to the limited period of time during which CS can be transferred by lymph nodes cells. As both CS and its passive transfer require γδ cells (ref. 21 and the present findings), the strong likelihood is that 4 days after immunization γδ cells leave the lymph nodes and spleen and localize in the peritoneum or bone marrow. The old observation that CS can be transferred by bone marrow or peritoneal cells taken at day 9 afer immunization[24,25] strongly supports this possibility.

Similar results have been reported in a previous paper by Ptak *et al.*[26] who showed that Vγ3$^+$ T lymphocytes are necessary for the passive transfer of CS. However, these authors found that the Vγ3$^+$ cells were active whithout immunization and rearranged the Vδ2 chain, suggesting that the the Vγ3 population might be indeed functionally and structurally heterogeneous.

In fact, the use of a particular TCR rearrangement appears to be a feature of mucosal γδ T lymphocytes (reviewed in ref. 27). DETC are characterized as a very homogeneous population since the vast majority of them express invariant T-cell receptor composed of Vγ3/Jγ1/Cγ1 and Vδ1/Dδ2/Jδ2/Cδ gene segments with no junctional diversity.[28] They show a broad reactivity toward keratinocytes treated with several different contact sensitizers which is apparently not MHC restricted but is mediated by the TCR.[29] Hence, it is probable that contact sensitizers evoke a common mechanism in keratinocytes and DETC have the capacity to respond to stressed cells in their vicinity. In addition, a possible role of cytokines, such as the keratinocyte-derived IL-7,[30] cannot be excluded. IL-7 has been suggested to sustain survival and growth of DETC[31] and is responsible for the migration of DETC to the draining lymph nodes after epicutaneous sensitization with TNCB.[21] Despite a wide range of different specificities described for human and murine γδ cells, there is little knowledge on the nature of the antigen they recognize. The lack of diversity in TCR expressed by Vγ3$^+$ T lymphocytes implies that the ligand recognized by these receptors is highly restricted. Asarnow *et al.*[28] have suggested that Vγ3$^+$ T lymphocytes recognize heat-shock proteins that are expressed following chemically induced stress, although Havran and Boismenu[32] showed that the stimulatory molecule was a low molecular weight fraction of acid-eluted material from stressed keratinocytes.

The vast majority of human peripheral γδ T cells, which express Vγ9 and Vδ2 TCR genes, recognize low molecular weight (< 500), protease-resistant ligands that contain critical phosphate moieties.[33–35] These ligands have been shown to be present both in bacterial and in mammalian cells.[36] The γδ T lymphocytes are scarce or not found at all in human skin. However, in allergic contact dermatitis to dinitrochlorobenzene,[37] and gold chloride and mercury chloride,[38] γδ cells were observed both in the epidermis and dermis and accounted for 15% of the whole CD3$^+$ cells. Interestingly, most of the infiltrating γδ cells expressed the Vγ9 and Vδ2 TCR gene segment, suggesting that they had in fact migrated from the peripheral blood. These results therefore indicate that γδ cells may play a role also in contact sensitivity reaction in humans.

Overall, the present results confirm the important role of Vβ8$^+$ and γδ$^+$ T lymphocytes in the CS reaction to the hapten TNCB and in its passive transfer, and give rise to the possibility that the time–course of the γδ$^+$ T lymphocytes may determine the time–course of the transfer of the CS reaction.

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