Changes in Delayed-type Hypersensitivity and Helper Function Activities of T Cell Lines and Clones during Long-term Culture*

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SUMMARY T cell lines were established from the lymph node cells of BALB/c mice which were immunised with a trintrophenyl (TNP) hapten. Most of these lines showed activities for both TNP-specific delayed-type hypersensitivity (DTH) and helper function. These activities of the cell lines were eliminated by treatment with anti-Lyt 1 but not anti-Lyt 2 antibody plus complement. The relative strength of DTH versus helper function was different from line to line. Of several clones established from a single line, one designated clone-2 retained both helper and DTH activities up to 5 months after the cloning. One month thereafter, however, one subculture of clone-2 selectively lost helper activity, whereas another subculture lost DTH activity. These results suggested that a single cell was able to mediate both DTH and helper functions, but that these functions represented distinct activities of the cell.

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Antibody response and delayed-type hypersensitivity (DTH) are entirely different phenomena, and the optimal conditions of immunisation for each are usually different with respect to antigen dose, adjuvant and route of antigen administration. In many instances, the generation of antibody response and DTH seem antagonistic.[1,2] We have shown previously that immunisation of mice by the method most effective in inducing helper activity, did not result in the generation of DTH.[3] On the other hand, recent studies have shown that the characteristics of T cells mediating helper function and T cells mediating DTH are virtually identical. Both cells belong to the Lyt 1^+2^- subset; both recognise antigen together with self Ia antigen; and both have no killer activity.[4-7] Thus it is commonly thought that a single T cell could mediate both phenomena. The finding of Bianchi *et al*[8] that a sheep erythrocyte specific T cell clone mediates both helper and DTH activities strongly supported the above idea. The apparent inverse relationship between helper and DTH activities is suggested to be attributable to the effect of suppressor cells working on distinct activities of the same cell,[9] though the exact mechanism of discriminant suppression has never been clarified.

We tried to investigate whether Lyt 1^+2^- cells were always capable of mediating both activities or whether they could differentiate to express one or the other activity depending upon the environmental conditions. Trinitrophenyl (TNP)-specific T cell lines and clones were established, and the helper and DTH activities were investigated during long term culture. We hypothesised that a T cell originally showing both activities might subsequently differentiate to express only one of them.

MATERIALS AND METHODS

Mice

BALB/c mice were obtained from the breeding colony of our In-

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stitute and were used at 10-12 weeks of age.

Immunisation

Mice were immunised subcutaneously at the base of the tail with $5x10^7$ X-irradiated (3,300 R) TNP-coupled syngeneic spleen cells which were suspended in complete Freund's adjuvant according to the method of Martinez-Alonso *et al.*[10] Mice were also immunised by painting the shaved abdomen with picryl chloride (PCl) as previously described.[11]

Hapten-coupled cells

A suspension of spleen cells were prepared as previously described,[12] and red cells were lysed by treatment with Gey's solution. Haptenation of spleen cells was performed according to the method of Martinez-Alonso *et al.*[10] Briefly, $5x10^7$ cells were suspended in 1 ml of Hanks' balanced salt solution containing 10 mM (for *in vivo* immunisation and for *in vitro* stimulation) or 3 mM (for helper assay) of 2,4,6-trinitrobenzene sulfonic acid (Nakarai Chemicals, Kyoto, Japan). Cells were incubated at 37°C for 10 minutes and washed 4 times with Eagle's minimum essential medium (MEM, Nissui Seiyaku Co., Tokyo, Japan).

Anti-Thy 1 and anti-Lyt antibodies

Monoclonal anti-Thy 1.2 antibody (clone F7D5) was purchased from Olac Ltd. (Oxon, England). Monoclonal antibodies specific for mouse Lyt 1.2 or Lyt 2.2 and Low-Tox-M rabbit complement (C) were purchased from Cederlane Laboratories Ltd. (Ontario, Canada). Methods for the treatment of cells with these antibodies and with C were as previously described.[13]

Long-term culture and cloning of T cells

The *in vitro* enrichment of TNP-specific T cells was performed by the method of Martinez-Alonso *et al*[10] with a slight modification. Four days after immunisation, the inguinal and para-aortic lymph nodes were removed and teased through 80-gauge stainless steel mesh. Cells were then passed through a nylon wool column as previously described.[12] Then, $5x10^6$ nylon-nonadherent cells were cultured with $30x10^6$ irradiated (3,300 R) TNP-coupled spleen cells in 5 ml of culture medium in a 50 ml culture flask (Nunc, Roskilde, Denmark). Every 7 days, cultures were fed with 3 ml of fresh medium containing $2x10^7$ irradiated TNP-coupled spleen cells. Culture medium was RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 24 mM of N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES, Nakarai Chemicals), 2 mM glutamine, 10 µg/ml of gentamicin (Sigma Chemical Co., St. Louis, Mo.) and 5% foetal calf serum (FCS, M.A. Bioproducts, Walkersville, Md.)

After culturing for 4 weeks, cells were harvested and the activities of helper and DTH function were tested. For cloning of T cells, a portion of the harvested cells were treated with anti-Lyt 2.2 antibody plus C. Cells were then plated at 0.3 cells/well in a microtest plate (Costar Division, Data Packaging Corp., Cambridge, Mass.) and cultured for 3 weeks in the presence of 3,300 R irradiated TNP- coupled spleen cells and the supernatant (10%) of a concanavalin A (Con A) -stimulated rat spleen cell culture as a source of T cell growth factor (TCGF). The method of preparation of rat Con A supernatant and the removal of Con A with Sephadex G-100 was as described by Watson.[14]

Assay for helper and DTH activities

The helper activity of T cell lines and clones was assessed by the method of Martinez-Alonso *et al.*[10] Various numbers of T cell lines or clones were cultured together with $5x10^4$ TNP-coupled or uncoupled splenic B cells (anti-Thy 1.2 plus C treated syngeneic spleen cells) in a 0.2 ml volume of RPMI 1640 medium supplemented with $5x10^{-5}$ M 2-mercaptoethanol, 10 mM HEPES and 10% FCS. In all experiments, TNP-coupled or uncoupled B cells were also cultured alone or in the presence of lipopolysaccharide (LPS 055:B5, 25 µg/ml, Difco Laboratories, Detroit, MI.). The number of Ig-producing cells was determined 5 days after culture initiation by the protein A plaque method as previously described.[13]

DTH activity of cultured cells was assayed by the local cell transfer method. Various numbers of T cells from the lines or clones to be tested were mixed with $1.5x10^7$ TNP-coupled spleen cells and injected subcutaneously into the hind footpads of normal BALB/c mice. Control groups of mice received cultured cells or eliciting antigen only. The estimation of footpad swelling was the same as reported in a previous study.[15]

RESULTS

Helper and DTH activities of T cells lines

T cell lines were established from the lymph node cells of BALB/c mice which were immunised with TNP-coupled spleen cells or with PCl. Cell lines (see Table 1) TNP-1 to TNP-6 were established from mice immunised with TNP-coupled spleen cells; PCl-1 to PCl-3 were from mice immunised with PCl. Helper and DTH activities of these cell lines were repeatedly assayed, and Fig. 1 shows representative results obtained with lines TNP-4 and PCl-2. As shown in Fig. 1, TNP-4 induced marked Ig production in TNP-coupled B cells but not in normal B cells, whereas PCl-2 induced only a slight Ig production. In contrast, DTH eliciting activity of PCl-2 was higher than that of TNP-4 (Fig. 2). It should also be noted that, on a per cell basis, these helper and DTH activities were about 10 times higher than those of antigen-primed uncultured lymph node cells (data not shown).

Table 1 Helper and DTH activities of 9 independently cultured lines

Cell lines	Immunisation of mice §	Activities of cell lines*			
		Initial culture #		TCGF culture †	
		Helper	DTH	Helper	DTH
TNP-1	TNP-spleen	++	ND		
TNP-2	TNP-spleen	++	+		
TNP-3	TNP-spleen	–	+	–	ND
TNP-4	TNP-spleen	++	+	++	ND
TNP-5	TNP-spleen	+	ND	+	+
TNP-6	TNP-spleen	++	++	+	+
PCl-1	PCl	+	ND		
PCl-2	PCl	+	++		
PCl-3	PCl	+	++		

* The strength of the activity for helper and DTH is scored on an arbitrary basis.
Helper activity: -, mean ± SE of experimental group overlaps with that of the control group which is the response of normal B cells cultured with the cell lines. +, mean number of PFC of the experimental group is 2 to 10 times higher than that of control group. ++, mean number of PFC of experimental group is > 10 times higher than that of control group.
DTH activity: -, mean ± SE of footpad swelling of experimental group overlaps with that of the control group which received TNP-coupled spleen cells only. +, mean values of footpad swelling is less than 7×10^{-1} mm. ++, mean value of footpad swelling is more than 7×10^{-1} mm.
\# Tested 4 weeks after the initiation of culture.
† Some of the lines were further cultured for 4 weeks in the presence of TCGF, and the activities were determined.
§ Cell lines were obtained from mice immunised with subcutaneous injection of TNP-coupled syngeneic spleen cells (TNP-spleen) or by painting with picryl chloride (PCl).

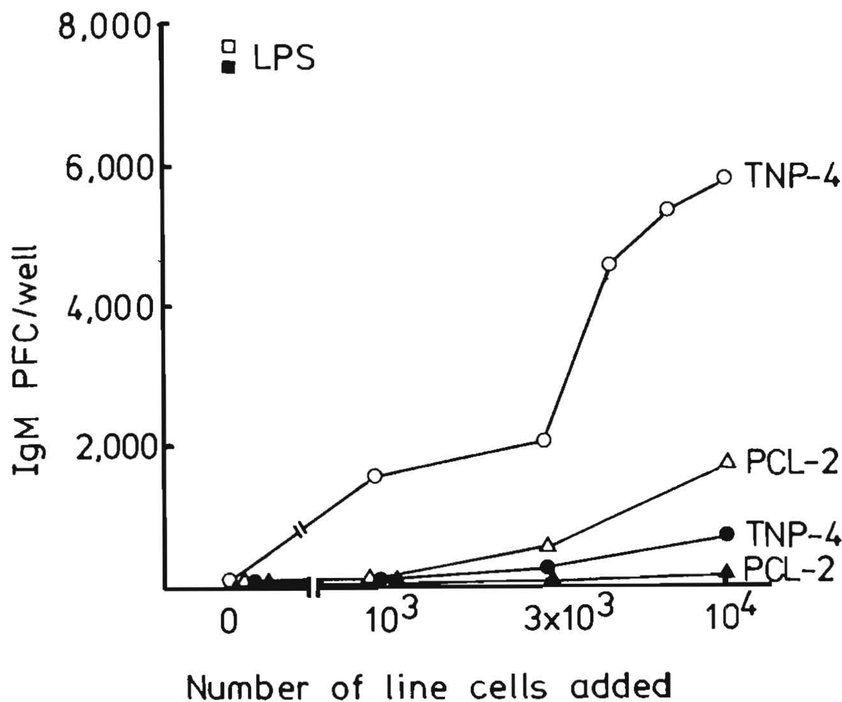

Fig. 1 Helper activity of TNP-4 and PCl-2 lines. TNP-4 and PCl-2 cells were harvested on the 4th week of culture. Five x 10^4 TNP-coupled B cells (open symbol) or uncoupled B cells (closed symbol) were cultured with various numbers of TNP-4 (○, ●) or PCl-2 (△, ▲) cells or with LPS (□, ■). IgM secreting cells were enumerated on day 5, and the arithmetic mean of triplicate culture is shown.

Lyt phenotype of T cells which mediate helper and DTH activities

On the 5th week of culture, cells of TNP-4 and PCl-2 were harvested and they were treated with C alone, anti-Lyt 1.2 plus C, anti-Lyt 2.2 plus C or anti-Thy 1.2 plus C. After the treatment, 6×10^3 viable cells were cultured with 5×10^4 TNP-coupled or uncoupled B cells to assess the helper activity. The numbers of Ig-producing cells were determined 5 days later. For the DTH assay, 10^5 viable cells were mixed with 10^7 TNP-coupled spleen cells and injected into the hind footpad of normal mice. Footpad swelling was measured 20 hours later. Fig. 3 shows that helper activity of these 2 lines was completely abolished by anti-Thy 1.2 or anti-Lyt 1.2 plus C treatment, whereas anti-Lyt 2.2 plus C treatment did not affect the helper activity. In Fig. 4, DTH activity of these lines was also shown to be abolished by anti-Thy 1.2 or anti-Lyt 1.2 but not anti-Lyt 2.2 plus C treatment. These results indicate that cells which mediate these two activities carry the Thy 1^+ Lyt 1^+2^- phenotype. Similar results were obtained with other cell lines (data not shown).

DTH versus helper function of cell lines

Although the TNP-4 and PCl-2 cell lines showed both helper and DTH activities, it was found that the relative strengths of these activities varied from line to line. Helper and DTH activities of all cell lines tested are summarized in Table 1 where the strengths of these activities are shown in arbitrary grades. Before cultivation with TCGF, 8 out of 9 lines showed both helper and DTH activities, whereas one line (TNP-3) expressed DTH activity only. The line PCl-3 selectively lost helper activity after prolonged culture with TCGF. It should also be noted that there was a tendency for the helper activity of cell lines from mice immunised

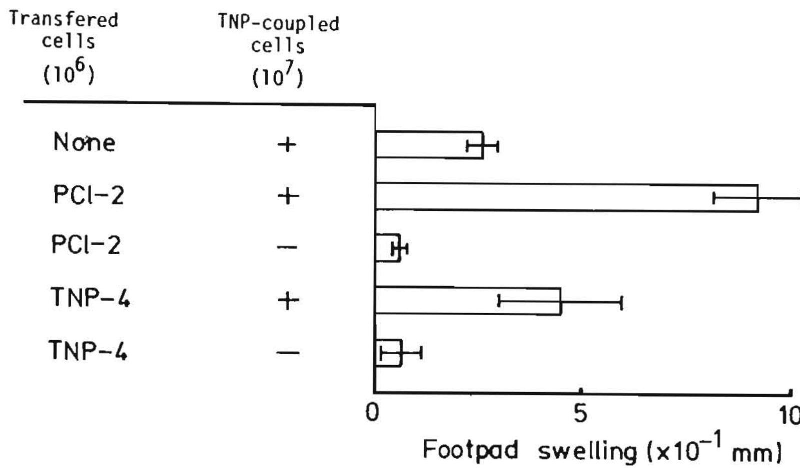

Fig. 2 DTH activity of TNP-4 and PCl-2 cells. TNP-4 or PC1-2 cells (10^6) were mixed with TNP-coupled spleen cells (10^7) and injected into the hind footpad of normal mice. Control groups received cultured cells or TNP-coupled spleen cells only. Footpad swelling was measured 20 hours later. Arithmetic mean ± SE.

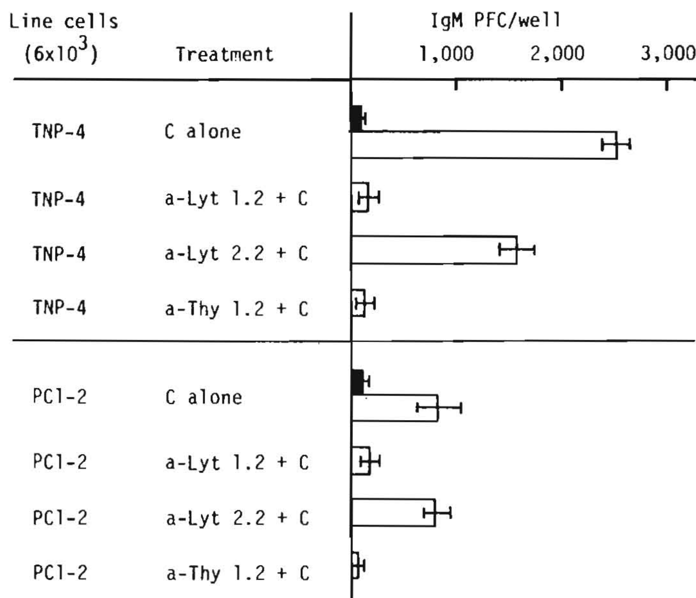

Fig. 3 Effect of anti-Lyt antibodies on helper activity of cell lines. TNP-4 and PC1-2 cells were harvested on the 5th week of culture and treated with C alone, anti-Lyt 1.2 plus C, anti-Lyt 2.2 plus C or anti-Thy 1.2 plus C. Six x 10^3 treated cells were cultured with $5x10^4$ TNP-coupled B cells for 5 days, and IgM PFC were enumerated. The solid column represents the response of normal B cells cultured together with cell lines treated with C alone.

with TNP-spleen cells to be higher than that of lines from PCl painted mice. The reverse was true for DTH activity.

Establishment of cloned lines

Cells of TNP-6 which showed high levels of helper and DTH activities were cultured in the presence of TCGF for 4 weeks and were cloned by limiting dilution as described in Materials and Methods. Two weeks after cloning, cell growth was observed in 6 of 96 wells. Four weeks after cloning, the helper activities of these clones were assessed by culturing 10^4 cloned cells with $5x10^5$ TNP-coupled or uncoupled B cells. The number of Ig-producing cells was determined on the 5th day of culture. Fig. 5 shows that 4 out of the 6 clones retained helper activity, whereas the remaining 2 clones did not in this assay. None of the clones showed significant helper activity against TNP-coupled B cells from CBA or C57BL/6 mice (not shown).

Dual function of one clone and the change of activity during long-term culture

Clone-2 cells, which showed the highest helper activity in the previous experiment (Fig. 5) were tested for helper and DTH activities on the 6th week of culture. The results shown in Fig. 6 indicate that this clone had both activities. The dual function of this clone had been retained in a subculture for up to 5 months after cloning (data not shown).

Two other subcultures of clone-2 were maintained independently and the cells of these subcultures were assayed for their helper and DTH activities 6 months after cloning. One subculture of clone-2 selectively lost helper activity (Fig. 7) while the other subculture lost DTH activity (Fig. 8). These results strongly suggest that a single cell is able to mediate both DTH and helper functions, but that these functions represent distinct activities of that cell.

DISCUSSION

The present study demonstrated that a TNP-specific T cell clone mediated both DTH and helper functions. The characteristics of this clone are quite similar to those of sheep erythrocyte specific clones reported by Bianchi *et al,*[8] and by Marchal *et al.*[16] On the other hand, Dennert *et al*[17] established a Lyt 1^+2^- clone which recognised an allogeneic Ia antigen and expressed DTH, helper and cytotoxic activi-

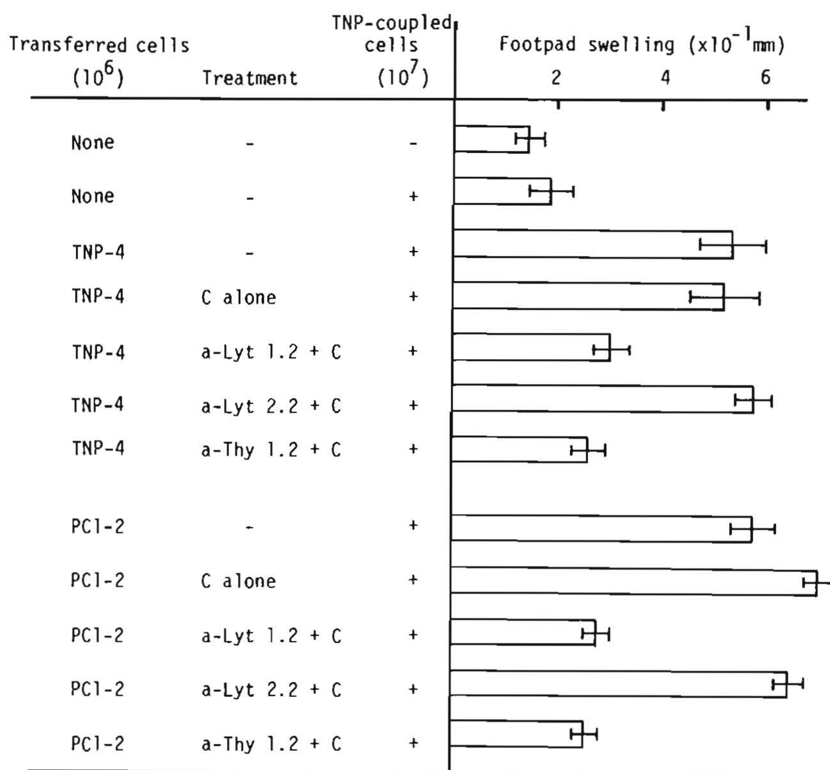

Fig. 4 Effect of anti-Lyt antibodies on DTH activity of cell lines. TNP-4 or PCl-2 cells were treated with antibodies as in Fig. 3, and 10^6 cells were mixed with 10^7 TNP-coupled spleen cells. The mixture was injected into the hind footpad of normal mice. Footpad swelling was measured 20 hours later.

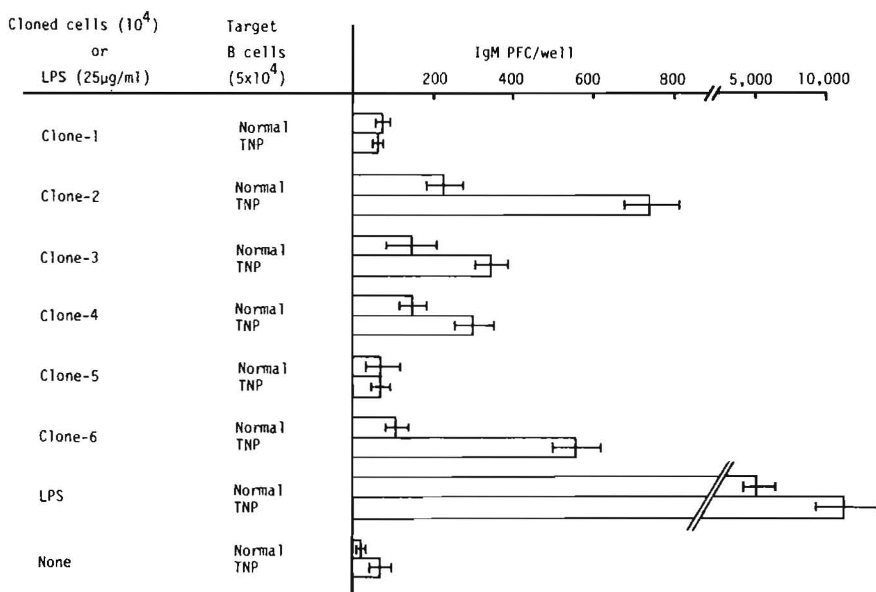

Fig. 5 Helper activity of clones derived from TNP-6 line. Four weeks after cloning, 10^4 cloned cells were cultured with $5x10^4$ TNP-coupled or uncoupled B cells. IgM secreting cells were enumerated on day 5. As controls, B cells were cultured alone or in the presence of LPS.

ties. Our TNP-specific clone (clone-2) is different in nature from this allo-specific clone, since it did not express killer activity (data not shown).

It is of fundamental importance to ask whether cloned cells are physiologically normal cells. It is probable that only cells of highly proliferative capacity and special characteristics can survive in long-term culture, or during such culture cells may change their characteristics. In conventional *in vitro* or *in vivo* systems, killer T cells are shown to be Lyt 2^+, whereas T cells mediating helper or DTH activity are Lyt 2^-. Thus, the allo Ia reactive T cell clone of Dennert *et al*[17] does not seem to fall within the normal classification of functional T cells, but might have derived from a multifunctional precursor of T cells. On the other hand, since all the characteristics thus far investigated of helper T cells and DTH-mediating T cells are the same or quite similar, clones mediating both these functions tend to be accepted as solid evidence supporting the idea that a single T cell is active in both functions. Before accepting this idea, however, it is necessary to reconcile observations on cell clones with *in vivo* findings that DTH and helper activity do not necessarily develop in parallel[3] or that there exists an inverse relationship between DTH and antibody response or helper activity.[1,2] It has been suggested that such an inverse relationship might be attributable to the effect of suppressor cells which could preferentially suppress one of the activities,[9] although concrete evidence for such regulatory cells has not been shown. The present findings that T cell lines derived from PCl painted mice and those derived from mice immunised with TNP-spleen cells tended to preferentially express DTH and helper activity, respectively, and that a clone initially expressing both functions (clone-2) spontaneously changed to express one or the other function

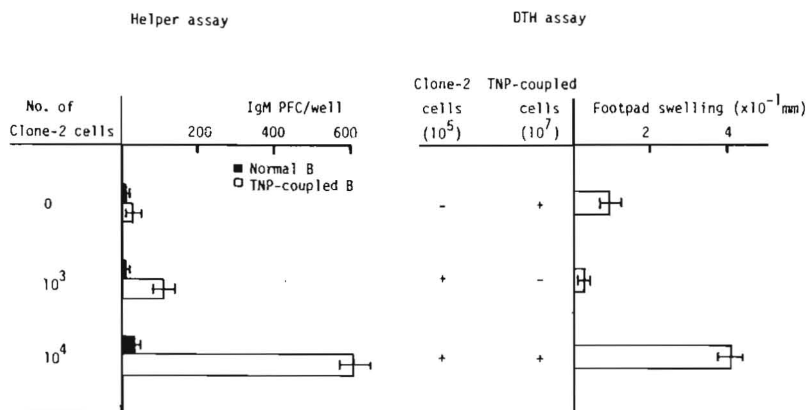

Fig. 6 Helper and DTH activities of clone-2 cells 6 weeks after cloning. Clone-2 cells (10^3 or 10^4) were cultured with $5x10^4$ TNP-coupled or uncoupled B cells for 5 days before enumerating the IgM secreting cells (Left). Clone-2 cells (10^5) were mixed with 10^7 TNP-coupled spleen cells and injected into the hind footpad of normal mice. Footpad swelling was measured 20 hours later (Right).

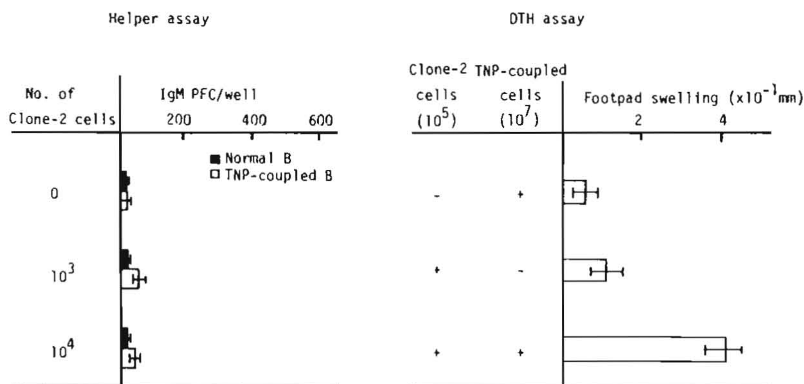

Fig. 7 A subculture of clone-2 (subculture-1) selectively lost helper activity 6 months after cloning. Subculture-1 cells (10^3 or 10^4) were cultured with $5x10^4$ TNP-coupled or uncoupled B cells and IgM secreting cells were enumerated on day 5 (Left). Subculture-1 cells (10^5) were mixed with 10^7 TNP-coupled spleen cells and injected into the hind footpad of normal mice. Footpad swelling was measured 20 hours later (Right).

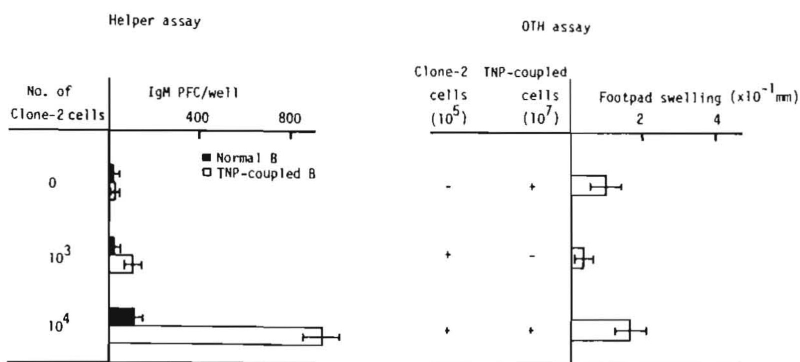

Fig. 8 A Subculture of clone-2 (subculture-2) lost DTH activity 6 months after cloning. Subculture-2 cells (10^3 or 10^4) were cultured with $5x10^4$ TNP-coupled or uncoupled B cells and IgM secreting cells were enumerated on day 5 (Left). Subculture-2 cells (10^5) were mixed with 10^7 TNP-coupled spleen cells and injected into the hind footpad of normal mice. Footpad swelling was measured 20 hours later (Right).

suggested the existence of such regulation. Further investigation on the mechanism(s) responsible for the change in activities of the T cell is required.

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