Antigenic requirements for stimulation and target cell recognition by immune and non-immune cytotoxic T lymphocytes

Article *in* Immunology and Cell Biology · September 1990

DOI: 10.1038/icb.1990.37 · Source: PubMed

CITATION
1

READS
38

6 authors, including:

Judith A Owen
Haverford College
29 PUBLICATIONS **436** CITATIONS

SEE PROFILE

Douglas Dorer
University of Tennessee Southern
23 PUBLICATIONS **1,520** CITATIONS

SEE PROFILE

Immunol. Cell Biol. (1990) **68**, 269–276

Antigenic requirements for stimulation and target cell recognition by immune and non-immune cytotoxic T lymphocytes

J. A. Owen, K. T. Grahl, A. Zador, D. R. Dorer, L. Klein and M. B. Rubin

Department of Biology, Haverford College, Haverford, PA 19041, USA

(Submitted for publication 6 March 1990. Accepted for publication 25 July 1990.)

Summary The molecular requirements for recognition of antigen-modified cells by cytotoxic T lymphocyte precursors (CTLp) and their activated progeny, cytotoxic T lymphocytes (CTL), have been compared using haptenated stimulator and target cells. The antigen density requirements of T cell recognition by fluorescein-specific CTLp and CTL derived both from naive mice and from animals previously primed *in vivo* were determined. The cell surface hapten concentration required to stimulate CTLp cannot be distinguished from that required on target cells for lysis by their mature daughter CTL 5–7 days later. However, if the CTL (and their precursor CTLp) are derived from mice primed *in vivo* with hapten-conjugated cells, they require lower cell surface hapten densities for recognition than do the analogous T cell populations from naive animals. Thus, the maturation of CTLp into CTL during 5–7 days *in vitro* does not result in any functionally relevant change in the nature or density of antigen receptors on the surface of the T cell. This is in contrast to the apparent selection which occurs over longer time periods *in vivo* following priming.

INTRODUCTION

Immunological memory is defined as the ability of the immune system to respond more effectively following a second as compared with a first exposure to antigen. Although an enhanced secondary response was first observed in studies of antibody production *in vivo* (1), it is now clear that some antigen-specific cytolytic T lymphocyte (CTL) populations also acquire many of the expected properties of a memory cell pool, such as an increase in the number of antigen-specific cells and in their apparent affinity for antigen (2,3). For example, the frequencies of cytolytic T lymphocyte precursors (CTLp) specific for the male H-Y antigen or viral antigens increase more than 10-fold following immunization (2,4–7), whereas the increase in frequency of allospecific CTLp after *in vivo* priming is much more modest (less than threefold) (2). In addition, there is an apparent increase in the proportion of high affinity CTLp after *in vivo* immunization (4,8).

A considerable amount of useful information about the CTL repertoire has been obtained by studies of the allogeneic response (2). However, a drawback of the allogeneic response as a model system for the investigation of T cell memory is that it is not possible to alter the target cell surface antigen concentration. A number of laboratories, therefore, turned to hapten-specific CTL systems in order further to explore the differences in antigenic requirements between primary and secondary CTL. The minimum hapten concentrations necessary for the stimulation of immune versus non-immune CTL were compared and the ability of secondary CTLp to be stimulated by lower concentrations of antigen than are required to trigger primary precursor cells was implied (9–11).

The purpose of our experiments was to further define the mechanisms which give rise to this apparent increase in the affinity of secondary as compared with primary CTL populations. We find that the change is dependent upon *in vivo* factors which are not reproduced in 5–7 day cultures *in vitro* even when every effort is made to optimize the help signals.

The fluorescein hapten model system was chosen in these experiments as the amount of

Correspondence: J. A. Owen, Department of Biology, Haverford College, Haverford, PA 19041, USA.

Abbreviations used in this paper: CTL, cytotoxic T lymphocyte; CTLp, cytotoxic T lymphocyte precursor; 5-FITC, 5-fluorescein isothiocyanate; TNBS, trinitrobenzenesulfonate; PBS, phosphate buffered saline; WM, washing medium or Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine serum; SDS, sodium dodecyl sulfate; Con A, concanavalin; i.p., intraperitoneal; i.v., intravenous.

hapten on the cell surface can be easily adjusted merely by altering the hapten concentration in the reaction solution and can readily be quantitated by flow cytometric measurements. Excess help was provided in the form of a concanavalin A-stimulated rat spleen cell supernatant (Con A SN) and the cultures were depleted of adherent cells in order to minimize the amount of antigen reprocessing which can occur *in vitro*. Finally, we have used the same cell type, the mastocytoma cell line P815, for the stimulation and lysis stages of our measurements in order to facilitate a more direct comparison of hapten density requirements for recognition by CTLp and their daughter CTL.

Using this experimental system, we were therefore able to eliminate alternative explanations for the differences between primary and secondary CTL populations which could potentially blur the interpretation of previous experiments of this nature. We eliminated the possibility of the main difference occurring at the level of the T helper rather than the cytotoxic T cell populations by maximizing the availability of T cell help during the 5–7 day *in vitro* culture period. However, it was not possible, by this strategy, to manipulate potential differences between primary and secondary T helper cells acting *in vivo* prior to excision of spleens for culture. In addition, we were not dependent on measuring only that subset of antigen-specific CTL having the highest affinity for antigen, since we compared the average avidity of our CTL populations, and not the minimum concentration of hapten necessary to facilitate lysis. Finally, we searched for, but failed to find differences between the recognitive capacities of CTLp and their daughter CTL.

MATERIALS AND METHODS

Mice

Six to eight week old Balb/c mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Antigens

5-Fluorescein isothiocyanate (5-FITC) was obtained from Molecular Probes, Junction City, Oregon or from Sigma Chemical Co., St Louis, MO. A fresh stock solution of 4 mg/mL in dimethylformamide was made up weekly. Immediately prior to cell conjugation, the stock solution was diluted in warmed phosphate buffered saline solution supplemented with 16 mmol/L borate (PBS borate) at pH 9·0.

Preparation of stimulator cells

P815 mastocytoma cells were used as stimulator and target cells throughout this series of experiments. Cells to be used as stimulators in bulk cultures were treated with 25 μg/mL of mitomycin C (Sigma Chemical Co, St Louis, MO) for 45 min at 37°C at a concentration of 10×10^6 cells/mL. They were washed twice with PBS enriched with 5% fetal bovine serum (washing medium or WM) and a third time with PBS pH 7·0. The cells were resuspended quickly in prewarmed PBS borate at pH 9·0 and an equal volume of warm PBS borate containing the appropriate concentration of 5-FITC was added. Where different concentrations of 5-FITC were used, the dilution series was made in dimethyl-formamide (DMF) so that the DMF concentration remained constant for every 5-FITC concentration. At the end of the incubation, a five-fold excess of cold PBS plus 0·5% bovine serum albumin was added to quench the reaction and the cells were quickly centrifuged. They were then washed three times in WM and counted.

Immunization protocol

Mice were immunized intraperitoneally (i.p.) with 10×10^6 mitomycin C treated spleen cells conjugated with a solution of 50 μg/mL 5-FITC. Control experiments compared the efficiency of priming i.p. versus intravenously (i.v.) and determined that there was no difference in priming efficiency between the two routes, that optimal priming was achieved with 10×10^6 spleen cells and that some enhancement of responsiveness could be achieved following immunization with as few as 300 000 cells. Animals were used as secondary donors no sooner than 3 weeks after *in vivo* priming.

Preparation of responder cells

Responder cells to be stimulated with P815s in cultures were first depleted of adherent cells in order to minimize reprocessing of the stimulating antigen by macrophages. The macrophage depletion was effected by passing each responder population through two successive Sephadex G10 columns pre-equilibrated with WM. Depleted cell populations were then washed and counted.

Set-up of cultures

Cultures were set up as described previously (13). In all experiments, the culture medium was further enriched by the addition of 25% Con A SN and α-methyl mannoside (10 mg/mL).

^{51}Cr Release assay

Chromium Release assays of CTL generated in bulk cultures were conducted exactly as described previously (13). Cell lysis is expressed as a percentage of ^{51}Cr release relative to non-specific spontaneous release in medium alone (SR) and total release in the presence of detergent (TR). Per cent specific lysis was calculated as per the formula:

$$\% \text{ specific } {}^{51}\text{Cr release} = \{(ER-SR)/(TR-SR)\} \times 100$$

where ER is the ^{51}Cr release measured in the experimental wells.

Preparation of Con A SN

Con A SN was prepared precisely as described previously (5).

Determination of the per cent of monocytes in the responder cell population pre- and post-Sephadex G10 passage

Samples of 5×10^5 spleen cells were labelled by standard procedures prior to passage through Sephadex G10, following one passage and after two successive passages through a G10 column, with monoclonal antibodies to the following antigens: F-480, a mouse monocyte/macrophage-specific antigen (15) and MAC1 (an antigen known to be present on monocyte/macrophages, but also on granulocytes) (16). Flow cytometric analysis was done on an Epics V flow cytometer sorter (Coulter Electronics Inc. Haileah, FL). The cytometer was equipped with an argon laser set at 500 mW power and 488 nm excitation. Orange fluorescence was collected through a 570 nm long pass filter. The per cent of cells staining with each of the markers was compared with the per cent of the positive cells in the negative control, stained only with the phycoerythrin-labelled second antibody.

Determination of the number of fluorescein molecules bound to the surface of P815 cells following conjugation with each of two different 5-FITC concentrations

Samples of P815 cells were treated with PBS borate pH 9·0 containing 0 (control), 8 and 20 μg/mL of 5-FITC under the usual conditions. Following three washes, the cells were fixed with 2% paraformaldehyde in PBS BSA azide. Green fluorescence was measured as previously described and collected through a 525 nm bandpass filter. The mean green fluorescence channel of each cell population was determined and compared with the mean fluorescence channels of standard beads conjugated with known numbers of fluorescein molecules per bead. (Flow Cytometry Standards Corporation, P.O. Box 12621 Research Triangle Park, NC). The mean number of fluorescein molecules bound per cell at the two different fluorescein concentrations was interpolated.

Analysis of data from antigen concentration experiments with bulk cultures

By titrating the concentrations of 5-FITC used in the conjugations of the stimulator or of the target cells populations, curves of per cent specific lysis against the conjugating 5-FITC concentration could be generated at each effector–target ratio. When information regarding the antigen concentration requirements for target cell formation was being sought, CTL generated by stimulation with P815 cells conjugated with 200 μg/mL of 5-FITC were tested for their ability to lyse a set of targets treated with different concentrations of 5-FITC. The per cent specific ^{51}Cr release measured on control, unconjugated targets was subtracted from that obtained on each of the 5-FITC targets and then a curve of per cent specific ^{51}Cr release against conjugating 5-FITC concentration was plotted (Fig. 1). In experiments designed to study the hapten concentration requirements for stimulation of the CTLp, replicate cultures were set up in which responder cells were mixed with P815 cells conjugated with a range of 5-FITC concentrations. Following *in vitro* stimulation, each culture was assayed on both 5-FITC conjugated (200 μg/mL) and control targets. The per cent specific ^{51}Cr release measured on the control targets was then subtracted from that mediated on 5-FITC targets by the same effector population and a curve of per cent specific lysis against conjugating fluorescein concentration was constructed as before.

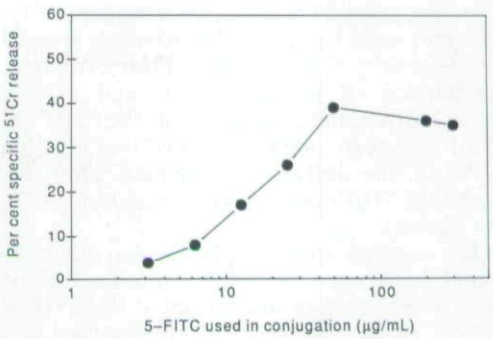

Fig. 1. Per cent specific ^{51}Cr release from target cells, conjugated with 5-FITC at the concentration shown on the *x*-axis. The per cent specific lysis of control, mock-conjugated targets by the same effector cell population has been subtracted from each value. The responder lymphocytes were splenocytes from unimmunized mice cultured *in vitro* for 5–6 days with mitomycin C-treated P815 cells conjugated with 200 μg/mL of 5-FITC.

For each such experiment, the concentration of conjugating 5-FITC that gave rise to half-maximal responsiveness was determined. This number is a useful measure of the average functional affinity of the CTL or the CTLp population under test. Half-maximal values were determined only when the curve of lysis versus 5-FITC concentration clearly plateaued at high 5-FITC concentrations (as in Fig. 1), and individual determinations of the conjugating concentration of 5-FITC giving rise to half-maximal lysis were made at each effector–target ratio that was tested.

RESULTS

Adherent cell-depleted CTLp respond to 5-FITC-conjugated P815 cells in the presence of a source of interleukin 2

5-FITC-conjugated P815 cells were used both as stimulators and as targets. In order to reduce the probability of antigen being reprocessed and presented to responding cells at effective concentrations quite different from those used in the original conjugation reaction, the re-

sponding splenic lymphocytes were depleted of adherent cells by successive passages through two Sephadex G10 columns. Flow cytometric comparison of unfiltered cells and of cells emerging from the columns, using the F/480 and Mac1 monocyte markers showed that less than 0·3% of the cells which remain after two Sephadex G10 passages were monocytes (data not shown).

The response measured in these experiments was H-2 restricted and 5-FITC specific. Little or no lysis was seen of unconjugated P815 ($H\text{-}2^d$) targets, of P815 cells covalently-coupled with the control hapten trinitrobenzene sulfonate or of EL4 ($H\text{-}2^b$) cells conjugated with 5-FITC.

In the early phases of these experiments Con A SN was routinely titrated to ascertain the optimal levels of the supernatant required in the culture. These varied between 15 and 25% for different batches of supernatant. Twenty five per cent Con A SN was therefore used in all the experiments described in this paper. Additional experiments demonstrated that 20 U/mL of recombinant interleukin-2 (IL-2) was capable of substituting for Con A SN in bulk cultures.

Antigen density requirements for stimulator and target cell recognition by unprimed CTL precursor cells and their immediate differentiated progeny are indistinguishable

An important and thus far unresolved issue is whether the CTL precursor and its activated CTL progeny share the same antigen concentration requirements for stimulation to differentiation, and for mediation of lysis of the appropriate target cells respectively. The hapten density requirements for stimulation of CTLp were compared with those for effective lysis by mature CTL, following 5–6 days of stimulation *in vitro*.

Fluorescein-specific CTL populations were generated *in vitro* by co-culture with P815 cells conjugated at 200 μg/mL of 5-FITC and were then tested for their ability to lyse a set of targets treated with different concentrations of 5-FITC.

Figure 1 shows an example of such a titration of per cent specific ^{51}Cr release versus increasing concentration of conjugating 5-FITC. Lysis measured on unconjugated target cells by the same effector population has been subtracted from each data point. Comparative values for the hapten density requirements for stimulation and target cell recognition were obtained by determining the concentration of conjugating 5-FITC that corresponded to half-maximal lysis of labelled P815 target cells at each effector–target cell ratio (see Materials and Methods). In the experiment of Fig. 1, the half-maximal lysis concentration was 12·5 μg/mL fluorescein. In experiments where titrations were performed at several effector–target cell ratios (usually 5:1, 10:1, 20:1 and 40:1), the half-maximal lysis concentration was found to be independent of effector–target cell ratios.

In a similar set of experiments, cells were incubated with mitomycin C-treated stimulator P815 cells which had been conjugated at different concentrations of 5-FITC. After 5–6 days in culture, the cells were harvested and the capacity of each population of cells to lyse targets conjugated at an optimal concentration of 200 μg/mL of 5-FITC and unconjugated control targets were measured. After subtraction of the control lysis values, curves of per cent specific ^{51}Cr release were constructed and half-maximal values of conjugating 5-FITC concentrations for stimulation were determined as before.

The upper half of Table 1 compares the conjugation concentration of 5-FITC necessary for half-maximal stimulation of naive CTLp with that required 5–6 days later for half-maximal lysis by fully differentiated CTL. These experiments demonstrate that the antigen density requirements for stimulation of CTLp are indis-

Table 1. Conjugating concentrations of 5-FITC resulting in half-maximal lysis by primary and secondary FITC-specific CTL.

CTL donor (naive or immunized)	Cells coupled to 5-FITC	Mean 1/2 maximal 5-FITC for CTL activity (μg/mL)*	Range of 1/2 maximal 5-FITC (μg/mL)
Naive	Stimulator	20·3	12·6–29·0†
Naive	Target	20·9	15·6–40·0
Immunized	Stimulator	8·2	4·8–11·2
Immunized	Target	8·4	7·0–10·2

*Interpolated from graphs as described in the text.

†Results represent data obtained in four (naive mouse, stimulator population), six (naive mouse, target cell population), three (immunized mouse, stimulator population), and two (immunized mouse, target population) experiments, respectively, at several different effector:target ratios per experiment.

tinguishable from those necesssary for target cell recognition by mature CTL derived from that precursor population.

Antigen density requirements for stimulator and target cell recognition by in vivo*-immunized CTL precursors and their progeny CTL are indistinguishable from one another, but differ from the analogous requirements exhibited by unprimed CTL(p)*

Comparable experiments were done to determine the hapten density requirements for stimulator and target cell recognition by CTLp and mature CTL derived from previously immunized mice. The results of these experiments are shown in the lower half of Table 1. The hapten concentration requirements for stimulator and target cell recognition were indistinguishable from one another. However, they were significantly lower than the corresponding values for a naive animal. Indeed, there was no overlap between the half-maximal values of 5-FITC concentrations required for stimulation or target cell formation for immune versus those for naive donors. (Student's *t*-test *P* values: Naive *vs* immune cell requirements for stimulation are the same; $P<0{\cdot}001$; naive *vs* immune cell requirements for target cell formation are the same; $P<0{\cdot}005$.)

These experiments clearly indicate that the portion of the CTL receptor repertoire that is specific for fluorescein in an immune mouse exhibits a type of 'affinity maturation' when compared with that found in a naive animal.

P815 cells conjugated at 8 and 20 μg/mL of fluorescein have demonstrably different levels of fluorescein bound to their cell surfaces

To this point, measurements of 5-FITC concentration had all been pertinent to the conjugating concentrations of 5-FITC in solution. Next, we determined how the amounts of fluorescein bound to the surfaces of the stimulator and target cell populations varied with the concentrations of fluorescein used in the conjugation reactions.

Cells were conjugated at the two concentrations of 5-FITC which gave rise to half-maximal recognition by primary and secondary CTL respectively. The cells were then fixed and analysed on a flow cytometer in order to determine the numbers of molecules of fluorescein bound per cell. The instrument was calibrated with control beads, which had known numbers of bound fluorescein molecules. Table 2 shows the results of two such experiments. The data show that, under these conditions, the mean number of fluorescein molecules per cell appeared to vary linearly with the hapten concentrations in the conjugating solutions.

Table 2. Flow cytometric measurement of the number of fluorescein molecules bound per P815 cell following conjugation of 8·0 or 20·0 μg/mL of 5-FITC.

Conjugating 5-FITC (μg/mL)	Mean integrated green channel	Calculated number of fluorescein molecules bound per cell ($\times 10^4$)
Exp. 1. 0·0	4	2·0
8·0	65	21·0
20·0	151	48·2
Exp. 2. 0·0	ND*	—
8·0	56	18·2
20·0	160	51·1

*ND = not done.

DISCUSSION

The experiments reported in this paper clearly demonstrate for the first time that there is no measurable difference in the hapten density required for effective stimulation of CTLp compared with that necessary for the recognition of susceptible target cells by their daughter CTL. This result was somewhat unexpected given the considerable differences between the size and the metabolic activity of CTLp and differentiated CTL.

For example, a decreased antigen concentration requirement for lysis compared with that needed for stimulation might have resulted from the elaboration of new surface receptors on the mature CTL induced by antigen- or lymphokine-induced triggering, resulting in a higher degree of effective receptor multivalency. A similar result might have been expected if the triggering event had caused an increase in the density of auxiliary structures such as Lyt2 molecules, which have been implicated in the process of antigen recognition, at least by some CTL clones (3,8).

Alternatively, an increased antigen concentration requirement could have been interpreted to suggest that, as the CTLp differentiated and increased in size, the synthesis of new receptor proteins fails to keep up with the increased surface area of the cell and hence the density of receptors would become reduced on the cell surface. The simplest interpretation of the present results is that the density of antigen receptor molecules remains relatively constant upon antigen-induced differentiation and, further,

that the same 'avidity' of interaction suffices to induce differentiation and to trigger the lytic mechanism.

We have also demonstrated that CTL and their precursors taken from immune mice can recognize cells conjugated with a lower cell surface hapten concentration than can CTL and CTLp from naive animals. Indeed, it was quite striking that the ranges of half-maximal concentrations of 5-FITC required for recognition by primary and secondary CTL, respectively, never overlapped.

The interpretation of earlier, provocative experiments leading to similar conclusions was hampered by the lack of appreciation, at the time they were conducted, of the need for T cell help in the generation of CTL activity (9,11). In addition the use of different cell types as stimulator and target cells in earlier work rendered difficult any comparison of the antigen density requirements for stimulator and target cell formation. In the current experiments, the antigen-presenting cells were P815 mastocytoma cells, which do not bear class 2 histocompatibility antigens, and which therefore cannot present antigen to class 2-restricted T helper cells. Excess T cell help is provided in these cultures in the form of Con A SN and the responder populations were depleted of monocytes before addition to the cultures.

It should be noted that each of these experiments entails a titration of stimulator or target cell hapten concentration followed by a determination of the conjugation concentration of 5-FITC required for half-maximal stimulation and/or lysis. The values obtained therefore, reflect an *average* 'avidity' of the CTL population being measured. In contrast, previous studies have focused on determining the minimal hapten concentration required for recognition and thus their results will reflect only the contribution of CTL with the highest 'avidity' for stimulator and target cells (9,10).

What might be the cellular and molecular correlates of the observed changes in the recognition requirements between primary and secondary fluorescein-specific CTL? Effects such as those described in this paper could result from: (i) clonal selection, such that those CTLp expressed in the primary response which bear receptor molecules with the highest affinity for antigen are preferentially expanded on stimulation and dominate the secondary response; (ii) somatic mutation of T cell receptor genes, with resultant selection by antigen for those receptors which can recognize lower antigen concentrations; (iii) the use of different receptor genes by primed as compared with unprimed T cells; (iv) an increase in the cell surface density of antigen receptors, or in the flexibility of their antigen binding sites; (v) an alteration in the ease with which a CTLp can be triggered or lytic activity can be induced in a CTL which is unrelated to the receptor molecule *per se*, but which may be influenced by the cell surface density of other, non-antigen specific moieties.

Each of these alternative interpretations can be subject to experimental analysis, following the generation of long-term CTL lines typical of primary and secondary fluorescein-specific responses. Our current thinking favours the first and simplest explanation of the observed results. Although some authors have suggested that the CTL antigen receptor might be particularly susceptible to frequent mutation (17,18), recent genetic data imply that extremely low levels of somatic mutation occur in T cell receptor genes, suggesting that the high levels of mutation found by others in *in vitro* systems may not accurately reflect the *in vivo* situation (19,20). We therefore consider it to be unlikely that the differences between the antigenic requirements for stimulation of primary and secondary CTL result from somatic mutation of the CTL receptor genes.

An additional possibility, that different receptor genes may be utilized by primary and secondary CTL has been suggested by reports of the analogous findings in a number of B lymphocyte responses (21,22,23). It has also been shown that differentiation from naive to memory T cells appears to be accompanied by increased expression of several surface molecules such as LFA-1, LFA-3, CD2 and Ly24 (3,24). Such an increase in cell surface antigen expression may render memory cells more susceptible to activation signals.

The development of B cell memory is usually associated with a marked increase in the frequency of antigen-responsive cells. A similar increase in the frequency of antigen-responsive cells upon *in vivo* immunization has been reported in the CTL response to influenza virus, murine sarcoma virus and to other antigens (4–7,25). It is, therefore, particularly interesting to note in the context of the present findings that, in preliminary experiments, the development of a memory response to this haptenic antigen did not appear to correlate with an increase in the frequency of hapten-specific CTL.

The interpretation of hapten density data in terms of their implication for T cell recognition is complicated by the fact that T cells must bind

to haptens which are covalently attached to residues of particular cell surface proteins, which in turn must be recognized in the context of histocompatibility antigens. The antigen that is being titrated as cell surface hapten density is altered is therefore a complex one and thus, not only the density, but also the nature of the antigenic determinants may be altering as more fluorescein is titrated into the conjugating solution. This caveat must be applied to any interpretation of such data in terms of possible increases in the affinity of interaction between primary and secondary CTL.

Acknowledgements The research described in this paper was supported by National Science Foundation RUI Grant number DCB 8410104 and by a Research Corporation Cottrell College Science grant. We thank Dr Meryle Melnicoff, Ms Colleen Jensen and Dr Katharine Muirhead for help with the flow cytometric analysis and Mr Russell Hill, Ms Kate Irvine and Dr Eric Nabors for assistance in some early experiments.

REFERENCES

1. Celada, F. 1971. The cellular basis of immunologic memory. *Prog. Allergy* **15**: 223–267.
2. Engers, H. D. and MacDonald H. R. 1976. Generation of cytolytic T lymphocytes *in vitro*. In *Contemporary Topics in Immunobiology*, W. Weigle (ed.) Plenum Press, New York. pp. 145–190.
3. Cerottini, J-C. and MacDonald, H. R. 1989. The cellular basis of T cell memory. *Ann. Rev. Immunol.* **7**: 77–89.
4. MacDonald, H. R., Cerottini, J-C., Ryser, J. E. *et al.* 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. *Immunol. Rev.* **51**: 93–123.
5. Owen, J. A., Allouche M. A. and Doherty P. C. 1982. Limiting dilution analysis of the specificity of influenza-immune cytotoxic T cells. *Cell. Immunol.* **67**: 49–59.
6. Owen, J. A., Allouche, M. A. and Doherty, P. C. 1984. Frequencies of influenza-responsive cytolytic T lymphocyte precursors in the thymus and spleen of unprimed mice. *Cell. Immunol.* **84**: 403–408.
7. Askonas, B. A., Mullbacher, A. and Ashman, R. B. 1982. Cytotoxic T memory cells in virus-infection and the specificity of helper T cells. *Immunology* **45**: 79–84.
8. MacDonald, H. R., Glazebrook, A. L., Bron, C., Kelsoe, A. and Cerottini, J. C., 1982. Clonal heterogeneity in the functional requirements for Lyt 2/3 molecules on cytolytic T lymphocytes (CTLs): possible implications for the affinity of CTL antigen receptors. *Immunol. Rev.* **68**: 89–115.
9. Pohlit, H., Haas, W. and von Boehmer, H., 1979. Cytotoxic T cell responses to haptenated cells. I. Primary, secondary and long term cultures. *Eur. J. Immunol.* **9**: 681–690.
10. Gilheany, P., Arora, P. K., Levy, R. B. and Shearer, G. M. 1981. H-2 linked genetic control of murine cell-mediated lympholysis to autologous cells modified with high and low concentrations of fluorescein isothiocyanate. *Cell. Immunol.* **59**: 97–105.
11. Moller, E. and Ramos, T. 1981. Comparison between the specificities of primary and secondary killer cells against alloantigens and hapten-modified syngeneic cells. *Scand. J. Immunol.* **13**: 127–141.
12. Fujiwara, H. and Shearer, G. M. 1980. Studies on *in vivo* priming of Tnp-reactive cytotoxic effector cell system II. Augmented secondary cytotoxic response by radioresistant T cells and demonstration of differential helper activity as a function of the stimulating dose of Tnp-self. *J. Immunol.* **124**: 1271–1276.
13. Owen, J. A., Scinto, L. A. F. S., Klein, L. and Kline, C. J. 1986. Cytolytic T lymphocytes discriminate between isomeric forms of the same hapten. *Immunology* **57**: 499–504.
14. Skinner, M. A., Prestridge, R. and Marbrook, J. 1985. The analysis of an anti-fluorescein response at limit dilution: The effect of lymphokine preparations on the apparent specificity. *Immunology* **55**: 463–471.
15. Austyn, J. M. and Gordon, S. 1981. F4.80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11**: 805–815.
16. Springer, T., Galfre, G., Secher, D. S. and Milstein, C. 1979. Mac-1: A macrophage differentiation antigen identified by a monoclonal antibody. *Eur. J. Immunol.* **9**: 301.
17. Reimann, J. and Miller, R. G. 1985. Rapid changes in specificity within single clones of cytolytic effector cells. *Cell* **40**: 571–581.
18. Weltzien, H. U., Kempkes, B., Jankovic, D. L. and Eichmann, K. 1986. Hapten-specific cytotoxic T cell clones undergo somatic variation of their antigen recognition specificity. *Eur. J. Immunol.* **16**: 631–639.
19. Fink, P. J., Matis, L. A. McElligott, D. L., Boohman, M. and Hedrick, S. M. 1986. Correlations between T cell receptor specificity and the structure of the antigen receptor. *Nature* **321**: 219–226.
20. Kumar, V., Kono, D. H., Urban, J. L. and Hood, L. 1989. T cell receptor repertoire and autoimmune diseases *Ann. Rev. Immunol.* **7**: 657–682.
21. Berek, C. and Milstein, C. 1987. Mutation drift and repertoire shift in the maturation of the

immune response. *Immunol. Rev.* **96**: 23–41.
22. Chang, S. P., Brown, M. and Rittenberg, M. B. 1982. Immunological memory to phosphorylcholine III. IgM includes a fine specificity population distinct from TEPC 15. *J. Immunol.* **129**: 1559–1562.
23. Dalesandro, M. R., Stein, S. L., Skeath, J. B. and Owen, J. A. 1990. Alterations in the U_H gene usage and fine specificity in primary and secondary FITC-specific IgM antibodies. *J. Cell. Biochem.* Suppl. 14D (abstr.)
24. Sanders, M. E., Makgoba, M. W., Sharrow, S. O. *et al.* 1988. Human memory T lymphocytes express increased levels of 3 cell adhesion molecules (LFA-3, CE2 and LFA-1) and three other molecules (UCHL1, CDW29 and Psp-1) and have enhanced IFNY production. *J. Immunol.* **140**: 1401–1407.
25. Rouse, B. T. and Wagner, H. 1984. Frequency of herpes simplex virus-specific cytotoxic T lymphocyte precursors in lymph node cells of infected mice. *Immunology* **51**: 57–64.