

# Generation and maintenance of autoantigen-specific CD8<sup>+</sup> T cell clones isolated from NOD mice

Laura Bowie<sup>a,\*</sup>, John Tite<sup>b</sup>, Anne Cooke<sup>c</sup>

<sup>a</sup> *Department of Medicine and Therapeutics, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland AB25 2ZD, UK*

<sup>b</sup> *Immunology Unit, Glaxo-Wellcome Medicines Research Centre, Stevenage, UK*

<sup>c</sup> *Department of Pathology, University of Cambridge, Cambridge, UK*

Received 26 February 1999; received in revised form 7 May 1999; accepted 12 July 1999

## Abstract

The non-obese diabetic (NOD) mouse develops insulin dependent diabetes mellitus (IDDM) spontaneously with a higher incidence in females than in males. There are many similarities to the human disease, making it an ideal model. Our group is examining the role that CD4<sup>+</sup> and CD8<sup>+</sup> T cells play in IDDM in the NOD mouse, as it is known that both T cell subsets are required for onset of disease. Although IDDM has an autoimmune etiology, the initial triggering event is unknown and the autoantigen involved has not been identified. This investigation focussed on one of the potential autoantigens involved, the enzyme glutamic acid decarboxylase (GAD). We raised GAD peptide-specific CD8<sup>+</sup> T cells by immunising NOD mice with the GAD peptide alongside an irrelevant peptide that induced a CD4<sup>+</sup> T cell response. In order to maintain these peptide specific T cells in vitro and generate clones, it was found that antibodies specific to CD4<sup>+</sup> and MHC class II molecules needed to be included in the culture medium. This paper outlines the methods we employed to generate and maintain these CD8<sup>+</sup> T cells in vitro. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** NOD mouse; CD8<sup>+</sup> T cells; IDDM; GAD

## 1. Introduction

Insulin dependent diabetes mellitus (IDDM) is an autoimmune disease resulting from the selective destruction of the insulin-producing  $\beta$  cells within the islets of Langerhans in the pancreas, leading to insulin deficiency. The non-obese diabetic (NOD) mouse develops IDDM spontaneously and is an ideal

model for the human disease. Unlike experimentally induced autoimmune diseases such as EAE and collagen-induced arthritis, the autoantigen in IDDM has not been established. The enzyme glutamic acid decarboxylase (GAD) which synthesises the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (Baekkeskov et al., 1990), is thought to be one of the most likely initiating autoantigens. Autoantibodies to GAD are found in the pre-diabetic human (Baekkeskov et al., 1987) and recent work has shown that induction of tolerance to GAD prevents or markedly reduces subsequent disease incidence in NOD mice (Kauf-

\* Corresponding author. Tel.: +44-1224-681818 ext. 53261; fax: +44-1224-273066; E-mail: l.bowie@abdn.ac.uk

man et al., 1993; Tisch et al., 1993). The objective of this study was to examine the role that CD8<sup>+</sup> T cells play in IDDM and the responses of this population to the potential autoantigen, GAD, were investigated. The strategy, therefore, was to investigate whether it was possible to induce cytotoxic T lymphocytes (CTL) specific for GAD from NOD mice and then examine the characteristics of these CTL.

Previous studies of the relationship of GAD to IDDM have focussed mainly on the 65 kDa isoform. However, this investigation centred on the 67 kDa isoform, as it has been shown to be more abundant in the murine pancreas, in contrast to humans and rats where it is the GAD<sub>65</sub> isoform which is more highly expressed in the islets (Kim et al., 1993; Petersen et al., 1993; Velloso et al., 1993). In order to induce GAD-specific CTL, mice were injected with peptides that were shown to bind to NOD MHC class I molecules.

Having obtained GAD peptide-specific cytotoxic cells from peptide-primed NOD mice, it was important to maintain these cells *in vitro* in order to characterise them and test their functional capacities. With spleen cells isolated from NOD mice there was the added problem of irrelevant autoreactive CD4<sup>+</sup> T cells overgrowing the desired cell line in culture: this has proven to be a general problem in this laboratory during the maintenance of CD8<sup>+</sup> T cells. The first step in this investigation was to find a way in which the GAD specific cell populations could be maintained *in vitro*, selecting for highly cytotoxic CD8<sup>+</sup>, CTL activity. This report outlines the method we have developed for maintaining peptide specific CD8<sup>+</sup> T cells *in vitro*.

## 2. Materials and methods

Chemical reagents were obtained from Sigma, Poole, UK, unless otherwise stated. Tissue culture plasticware was from Falcon, NJ, USA, unless stated otherwise.

### 2.1. Experimental animals

NOD mice were obtained from breeding colonies established at CRC, Northwick Park, London, and at

the Dept. of Pathology, University of Cambridge (UK). Mice were kept in the animal facilities of the Dept. of Pathology, University of Cambridge, Cambridge, with free access to food and water. All animals were housed and treated in accordance with the British Home Office regulations for the care and maintenance of animals for use in research.

### 2.2. Cells

The DBA/2 mastocytoma cell line P815 was maintained at Wellcome/Glaxo Research Labs, Kent, and EL4 cells were obtained from the ATCC. RMA-S cells were a gift from Dr. G. Butcher, while RMA-S cells transfected with the K<sup>d</sup> gene were a gift from Dr. F. Momberg, Heidelberg.

### 2.3. Medium

P815 cells were cultured in RPMI 1640 (Gibco BRL, Paisley, Scotland) medium supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 × 10<sup>-5</sup> M 2-mercaptoethanol; this is referred to as complete medium. Spleen cells from naive and primed mice were cultured in 10 ml of complete RPMI medium, with the addition of 20 IU/ml human IL-2 (Glaxo) with or without peptides (10 µg/ml). After several experiments, it was found that the addition of 10 ng/ml mouse IL-7 (Genzyme, MA, USA) helped to promote CD8<sup>+</sup> T cell growth and this was also included in the medium. For long term *in vitro* culture of spleen cells to select cytotoxic CD8<sup>+</sup> T cells, supernatants containing anti-CD4 (YTS 191) and anti NOD MHC class II (OX 6) antibodies were included at a final concentration of 20% in the complete medium supplemented with IL-2, IL-7, and peptides as described. The original antibody-producing cell lines, YTS 191 and OX6, were from Prof. H. Waldmann and Dr. D. Mason, respectively. The cell lines were grown in complete RPMI medium until confluent and then the supernatant was harvested. Although the amount of immunoglobulin in each preparation was not quantified, the supernatant was titrated to determine the optimal concentration for preventing the growth of CD4<sup>+</sup> T cells.

#### 2.4. Restimulation of cells *in vitro*

Cells that were maintained long term *in vitro* were restimulated weekly with  $5 \times 10^6$  irradiated, syngeneic antigen presenting cells (APCs), antigen, IL-2, IL-7, anti-class II and anti-CD4 antibodies as previously described in 25 cm<sup>2</sup> tissue culture flasks. The flasks were kept upright at 37°C in 5% CO<sub>2</sub>.

#### 2.5. T cell cloning

Clones were established from T cell lines by limiting dilution in 96-well, flat-bottomed plates, and cultured *in vitro* with antigen, IL-2, and IL-7, as previously described. One hundred cells/well were double diluted down to a concentration of approximately 0.3 cells/well, and  $5 \times 10^5$  syngeneic, irradiated APCs were added to each well. Proliferating cells were transferred to 24 well plates, then re-cloned in the same manner and restimulated weekly until clones were established.

#### 2.6. FACS analysis

FITC-labelled anti-CD4 and anti-CD8 antibodies were obtained from Becton Dickinson (Oxford, UK) and were used routinely for determining CD4<sup>+</sup>/CD8<sup>+</sup> levels in cell populations, such as those depleted of CD4<sup>+</sup> T cells. Tissue culture supernatants were also used to determine CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers; the original antibody-producing cell lines (YTS 191 and YTS 169, respectively) were a gift from Prof. H. Waldmann, Oxford. A panel of anti-Vβ monoclonal antibodies was used to determine Vβ usage of the T cell clones; these were used as undiluted tissue culture supernatants.

#### 2.7. RMA-S MHC class I binding assay

RMA-S or RMA-S-K<sup>d</sup> cells were incubated at 26°C overnight in order to induce surface expression of MHC class I. Peptides were then added to the cells at a concentration of 50 μg/ml for 3 h, after which time the surface expression of MHC class I was determined using FACS analysis. Peptides which bind to the MHC class I molecules will stabilise its expression (Ljunggren et al., 1990).

#### 2.8. Peptide immunisation

Initially, male NOD mice (> 6 weeks) were immunised with 100 μg GAD peptide mixed 1:1 with complete Freund's adjuvant (CFA) (Difco Labs, Detroit, USA) sub-cutaneously. However, after assessing several experimental strategies, it was found that the most successful way to generate peptide-specific CTL was to prime with GAD peptide together with a T helper peptide epitope, Ovalbumin (OVA) 323–339, mixed 1:1 (vol:vol) with incomplete Freund's adjuvant (IFA). Fifty microgram GAD peptide were mixed with 50 μg helper peptide. This peptide mixture was then mixed 1:1 with IFA and sonicated to achieve a good emulsion. Mice were immunised subcutaneously with 100 μg peptide and boosted intra-peritoneally with the same preparation 3 weeks later. After a further 3 weeks, spleens were removed, single cell suspensions prepared, and  $2 \times 10^7$  spleen cells were incubated with GAD peptide (10 μg/ml), IL-2 (20 IU/ml), and, latterly, IL-7 (10 ng/ml). These cells were harvested 5–7 days later and used as effector cells in cytotoxicity assays.

#### 2.9. Chromium release assays

Standard chromium release assays were used to test whether specific CTL had been generated by peptide immunisation. P815 (H-2<sup>d</sup>) and EL4 (H-2<sup>b</sup>) target cells were labelled with 100 μCi <sup>51</sup>Cr (Amersham International, Amersham, UK) per  $2 \times 10^6$  cells for an hour at 37°C in an equal volume of RPMI without FCS. Peptides were then added to the cells at a concentration of 50 μg/ml and the mixtures incubated for 30 min, before the cells were washed  $\times 3$  with PBS. Target cells were then added to 96-well u-bottomed plates together with effector cells to give the desired effector to target (E:T) ratio in a final volume of 200 μl of complete RPMI. Plates were then placed at 37°C for 6 h. Aliquots (100 μl) of supernatant were harvested and the radioactivity measured in a gamma counter. Specific lysis was determined using the formula:  $100 \times (\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{cpm maximum release} - \text{cpm spontaneous release})$ . Spontaneous release was calculated from wells containing only target cells in medium, and 100 μl of

detergent (1% Triton) were added to 100  $\mu$ l targets to determine maximum release. The standard error of the mean (S.E.M.) of triplicate cpm was never > 10% of the mean cpm.

### 3. Results

#### 3.1. Identification of potential GAD CTL epitopes

The motifs for binding to MHC K<sup>d</sup> and D<sup>b</sup> described by Falk et al., 1991 were used to predict potential CTL epitopes in the mouse GAD<sub>67</sub> sequence. A total of 13 sequences were identified: nine potential K<sup>d</sup> binding motifs and four potential D<sup>b</sup> binding motifs. After synthesis, these peptides were tested to determine whether they would bind to MHC class I molecules using the RMA-S stabilisation assay. Positive controls were peptides HPV E7(49–57) and NP(147–155) which bind to D<sup>b</sup> and K<sup>d</sup>, respectively. As shown in Table 1, seven of the GAD peptides bound to either D<sup>b</sup> or K<sup>d</sup> molecules. These seven peptides were then injected into NOD mice as described. As a control, mice were also immunised with the influenza peptide NP(147–155) which has been shown to induce K<sup>d</sup>-restricted CD8<sup>+</sup> CTL in other strains of mice (Taylor et al., 1987).

Table 1

Shown below are the peptides that were seen to bind to NOD MHC class I using the RMA-S and RMA-S K<sup>d</sup> binding assay described

<i>K<sup>d</sup> binders</i>	
C2	
W Y I P Q S L R G V	mouse GAD <sub>67</sub> 515–524
F3	
L Y V N A T A G T	mouse GAD <sub>67</sub> 338–346
B4	
S Y D T G D K A I	mouse GAD <sub>67</sub> 444–452
D4	
G Y Q P Q G D K A	mouse GAD <sub>67</sub> 554–562
<i>D<sup>b</sup> binders</i>	
B5	
G A I S N M Y S I	mouse GAD <sub>67</sub> 251–259
D5	
S V T W N P H K M	mouse GAD <sub>67</sub> 397–405
E5	
G D K A N F F R M	mouse GAD <sub>67</sub> 559–567

#### 3.2. No GAD CTL activity can be generated in NOD mice without the co-immunisation of a helper peptide

No lytic activity was detected in repeated chromium release assays using spleen cells from GAD-peptide injected and control-peptide injected mice as the effector cell population against peptide pulsed target cells, even at a high effector to target ratio (100:1).

#### 3.3. Co-immunisation with a helper epitope facilitated CD8<sup>+</sup> T cell priming

Since the above method was unsuccessful, the methodology described by Widmann et al. (1992) was therefore adopted using the peptide OVA 323–339, which has been demonstrated in this laboratory to induce CD4<sup>+</sup> T cell responses in NOD mice. Initially, ‘hybrid’ peptides consisting of the GAD peptides and a control peptide covalently linked to OVA 323–339 were injected into mice. However, no peptide specific CD8<sup>+</sup> T cells were primed using this method. Successful priming was found to occur when the GAD or control peptides were mixed separately with an equal amount of this helper peptide before being emulsified 1:1 with IFA as described. After priming and boosting with 100  $\mu$ g GAD peptide/OVA peptide, spleen cells were set up in culture for 5–7 days before being used as cytolytic effector cells in chromium release assays.

#### 3.4. GAD peptide-specific CTL can be induced by co-immunisation with OVA 323–339

Although the control peptide was able to induce an NP(147–155) specific T cell response, only one out of the seven GAD peptides used to prime NOD mice gave rise to specific CTL. This peptide was the K<sup>d</sup>-restricted peptide C2, corresponding to GAD<sub>67</sub> 515–524. Fig. 1 shows the percentage of peptide-specific and non-specific killing by spleen cells from C2-primed NOD mice as measured by chromium release. These peptide specific cells were then chosen for further analysis.

#### 3.5. C2-reactive cells lose their reactivity during *in vitro* culture

Spleen cells from C2/OVA(323–339) primed mice were prepared as single cell suspensions and

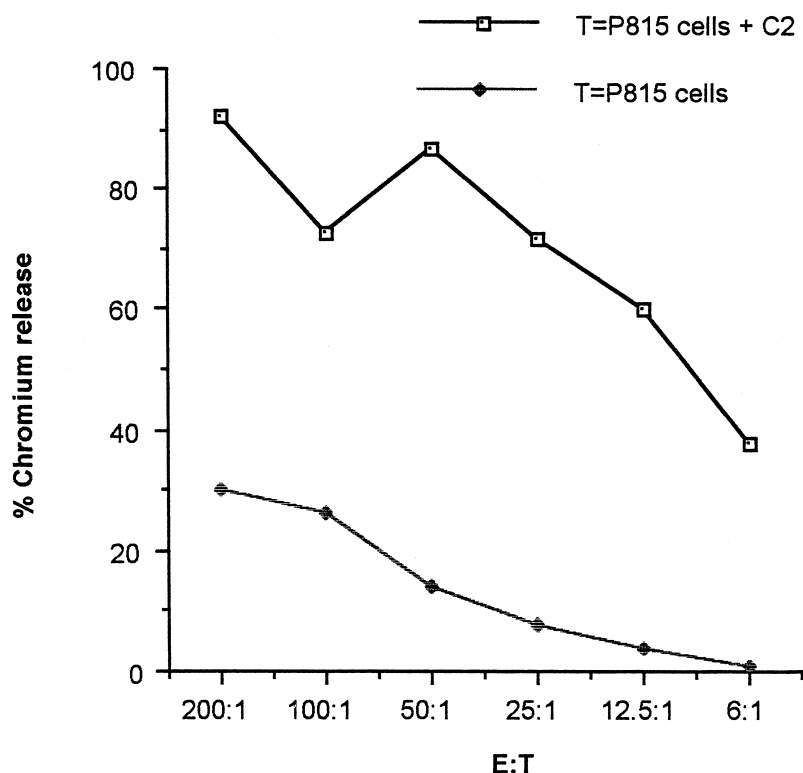


Fig. 1. After priming and boosting NOD mice with GAD peptides, together with a 'helper' peptide, only C2-specific CTL (mouse GAD<sub>67</sub> 515–524) were generated. Graph shows the percentage chromium release determined from the mean counts per minute (cpm) of triplicate wells, where the standard deviation (S.D.) of the triplicate cpm was < 10% of mean cpm. Three immunised mice were sacrificed and their spleen cells pooled, cultured in vitro for 5 days before acting as the effector (E) cell population against P815 target (T) cells. Target cells with or without exogenous peptide were incubated with the E cell population at the E:T ratios shown.

$2 \times 10^7$  cells cultured in 10 ml complete RPMI supplemented with OVA 323–339 and C2 peptide, each at 10  $\mu\text{g}/\text{ml}$ . After 5–7 days at 37°C, some of these spleen cells were harvested and used as cytolytic effector cells in chromium-release assays. The remaining cells were restimulated after 7 days with irradiated NOD spleen cells, IL-2 (20 IU/ml), and C2-peptide (10  $\mu\text{g}/\text{ml}$ ) as described in Section 2. Five days after restimulation, these cells were again tested for cytotoxic function against C2. It was found that cells that had been initially reactive against the GAD peptide lost this reactivity within 2 weeks. FACS analysis revealed that CD4<sup>+</sup> T cells had become the predominant cell population in the culture, and this was preventing the growth of C2-reactive, CD8<sup>+</sup> T cells. To try and prevent the growth of CD4<sup>+</sup> cells, spleen cells taken from C2-primed mice were depleted of CD4<sup>+</sup> T cells as soon as they were

obtained. Although the depletion was apparently very successful (only 0.49% of spleen cells were CD4<sup>+</sup> after depletion), these spleen cells still lost their cytotoxic potential after 2 weeks in vitro, demonstrating the particularly aggressive growth characteristics of any contaminating CD4<sup>+</sup> T cells.

### 3.6. Anti-class II and anti-CD4 antibodies in culture medium promote CD8<sup>+</sup> T cell growth

The most effective method of growing C2-specific cells long term was achieved by the inclusion of anti-CD4 and anti-class II monoclonal antibodies in the culture medium. Antibodies were included for repeated restimulations until CD4<sup>+</sup> T cells could not be detected by FACS analysis. IL-7 was also included in the medium at 10 ng/ml, as this cytokine has been shown to promote T cell growth in vitro

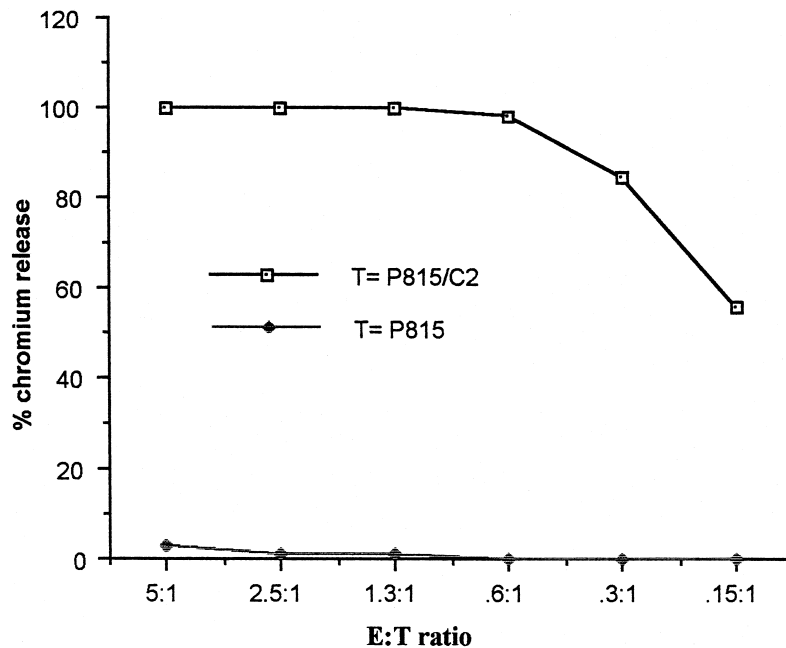


Fig. 2. The cytotoxic activity of C2 peptide-primed NOD spleen cells cultured with C2 *in vitro* for 7 weeks in the presence of anti-CD4 and anti-class II antibodies is shown. Without these antibodies present, all cytotoxicity disappears within 2 weeks of *in vitro* culture. Graph shows the percentage chromium release calculated from the mean cpm of triplicate wells, where E = effector cell population and T = target cell population. The S.D. of triplicate cpm was < 10% of the mean cpm.

(Chantry et al., 1989; Conlon et al., 1989; Okazaki et al., 1989; Everson et al., 1990). Using this protocol, a highly cytotoxic, C2-specific CD8<sup>+</sup> T cell line was obtained. After 7 weeks *in vitro* culture in the presence of the antibodies, the C2-reactive cells were cytotoxic at low E:T ratios, as shown in Fig. 2.

### 3.7. C2-specific clones are V $\beta$ 6<sup>+</sup> and highly cytotoxic

When CD4<sup>+</sup> T cells could no longer be detected in the long term culture of C2-specific spleen cells, limiting dilution in 96-well flat bottomed plates was performed to obtain clones. When the cells had reached sufficient cell density to permit transfer to tissue culture flasks, FACS analysis was performed to determine the T cell receptor V $\beta$  usage of cells that proliferated in response to C2 peptide. There were four potential clones determined in this way; all of them were V $\beta$ 6<sup>+</sup>, and CD8<sup>+</sup>. In fact, V $\beta$  staining of the original C2-specific cell 'line' revealed that it was also 100% V $\beta$ 6<sup>+</sup> suggesting that the line was

already clonal or oligoclonal. Using these cells as effectors in chromium assays revealed that all five of the clones were highly cytotoxic for C2-pulsed targets as shown in Fig. 3.

## 4. Discussion

As outlined, CTL activity can be reproducibly induced in NOD mice by immunisation with the GAD peptide C2 (which represents the sequence 515–524) if it is immunised in IFA together with a helper peptide. We have also employed this method for raising other peptide specific CTL in NOD mice. This confirms observations presented by others that the induction of a CTL response *in vivo* may require activation of a CD4<sup>+</sup> helper response, even if the CD4<sup>+</sup> T cell response is towards a different antigen (Wagner et al., 1976; Bennink and Doherty, 1978; Zinkernagel et al., 1978; Von Boehmer and Haas, 1979; Baum and Pilarski, 1981; Keene and Forman, 1981; Juretic et al., 1985; Kast et al., 1986; Husmann

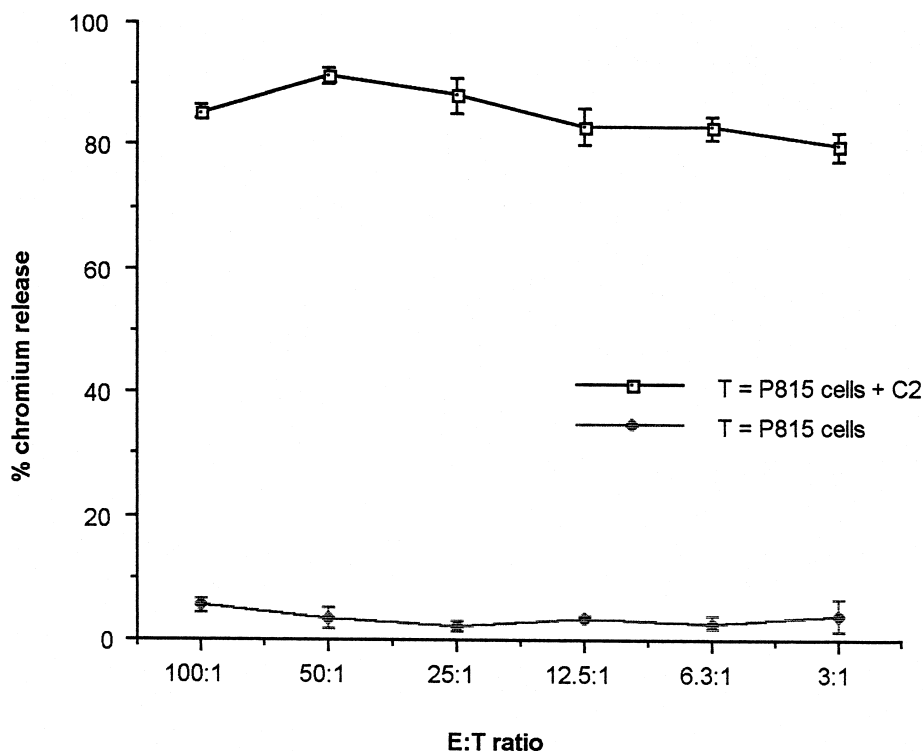


Fig. 3. The graph shows the percentage chromium release elicited by the five C2-specific clones when incubated with C2-pulsed target cells. Each clone was set up as the effector cell population in separate assays in triplicate wells, and the percentage lysis calculated from the mean cpm at each E:T ratio. The mean % chromium release of all five clones is shown, where the error bars are the standard error of the mean. E = effector cells and T = target cells.

and Bevan, 1988). The fact that we found that covalently linking a helper peptide to C2 prevented efficient priming of CD8<sup>+</sup> CTL is in contrast to observations by Widmann et al. (1992), who reported the use of this strategy in raising CTL specific for a malaria epitope in BALB/c mice. This may be due to differences in antigen processing and presentation between the two strains. NOD mice do not express I-E molecules but rather an unusual I-A, I-A<sup>g7</sup> (Acha-Orbea and McDevitt, 1987); the presentation of peptides by this class II molecule has not been well characterised.

The results discussed in this paper show that it is possible to maintain CD8<sup>+</sup> T cells from NOD mice *in vitro*, despite the initial difficulties encountered. The main problem to be overcome was to prevent the relatively aggressive CD4<sup>+</sup> autoreactive T cells from expanding and overcoming any CD8<sup>+</sup> T cell

responses. The addition of anti-CD4 and anti-class II antibodies in the medium successfully prevented the CD4<sup>+</sup> T cells in the spleen cell population from expanding. In this way, the C2-specific, CD8<sup>+</sup> T cells present were able to expand and become highly specific through repeated restimulations. We have also found that C2-specific cells can be isolated from non-primed diabetic NOD mice. However, the inclusion of anti-CD4 and anti-class II antibodies was required in the culture medium before a cell line could be maintained *in vitro*.

The cytokine IL-7 has been implicated in both B cell maturation (Namen et al., 1988a,b; Henney, 1989) and T cell development (Namen et al., 1988b; Montgomery and Dallman, 1991; Wiles et al., 1992) *in vivo*. *In vitro* it has been demonstrated to act synergistically with IL-2 in stimulating thymocytes (Chantray et al., 1989; Conlon et al., 1989; Okazaki et

al., 1989; Everson et al., 1990). Although the C2-specific clones were able to survive with just IL-2 as an added cytokine, the addition of IL-7 to the culture medium increased the proliferation rate of the cells; more cells could be harvested 5 days after restimulation, permitting earlier characterisation. Any cells cultured in vitro, either long term or for 5–7 days only, were therefore given IL-7 to promote T cell growth. The method described in this paper has been shown to be reproducible for generating CD8<sup>+</sup> T cell clones specific for GAD<sub>65</sub> epitopes, and is now used routinely in this laboratory.

### Acknowledgements

This work was supported by funding from the MRC, Wellcome-Glaxo, and the Wellcome Trust. The OVA helper peptide was described by R. Quartey-Papafio and Dr. N. Parish cultured the OX6 antibody.

### References

- Acha-Orbea, H., McDevitt, H., 1987. The first external domain of the nonobese diabetic mouse class II I-AB chain is unique. *Proc. Natl. Acad. Sci. (USA)* 84, 2435–2439.
- Baekkeskov, S., Landin-Olsson, M., Kristen, J.K., Srikanta, S., Bruining, G.L., Mandrup-Poulsen, T., de Beaufort, C., Soeldner, J.S., Eisenbarth, G., Lindgren, F., Sundkvist, G., Lernmark, A., 1987. Antibodies to a Mr 64,000 human islet cell antigen precede the clinical onset of insulin-dependent diabetes. *J. Clin. Invest.* 79, 926–934.
- Baekkeskov, S., Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., De Camilli, P., 1990. Identification of the 64 K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347, 151–156.
- Baum, L.L., Pilarski, L.M., 1981. The in vivo cytotoxic T cell response to alloantigen requires a Lyt 1+ helper T lymphocyte. *Transplantation* 32, 409.
- Bennink, J.R., Doherty, P.C., 1978. Different rules govern help for cytotoxic T cells and B cells. *Nature* 276, 829.
- Chantry, D., Turner, M., Feldmann, M., 1989. Interleukin 7 (murine pre-B cell growth factor/lymphopoietin 1) stimulates thymocyte growth: regulation by transforming growth factor beta. *Eur. J. Immunol.* 19, 783–786.
- Conlon, P., Morrissey, P.J., Nordan, R.P., Grabstein, K.H., Prickett, K.S., Reed, S.G., Goodwin, R., Cosman, D., Namen, A.E., 1989. Murine thymocytes proliferate in direct response to interleukin-7. *Blood* 74, 1368–1373.
- Everson, M.P., Eldridge, J.H., Koopman, W.J., 1990. Synergism of interleukin 7 with the thymocyte growth factors interleukin 2, interleukin 6, and Tumour Necrosis Factor  $\alpha$  in the induction of thymocyte proliferation. *Cell. Immunol.* 127, 470–482.
- Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., Rammensee, H.G., 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351, 290–296.
- Henney, C.S., 1989. Interleukin 7: effects on early events in lymphopoiesis. *Immunol. Today* 10, 170–174.
- Husmann, L.A., Bevan, M.J., 1988. Cooperation between helper T cells and cytotoxic T lymphocyte precursors. *Ann. N. Y. Acad. Sci.* 532, 158.
- Juretic, A., Malenica, B., Juretic, E., Klein, J., Nagy, Z., 1985. Helper effects required during in vivo priming for a cytotoxic response to the H-Y antigen in nonresponder mice. *J. Immunol.* 134, 1408.
- Kast, W.M., Bronkhorst, A.M., de Waal, L.P., Melief, C.J.M., 1986. Cooperation between cytotoxic and helper T lymphocytes in protection against lethal Sendai virus infection. Protection by T cells is MHC restricted. *J. Exp. Med.* 164, 723–738.
- Kaufman, D.L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G.S., Robinson, P., Atkinson, M.A., Sercarz, E.E., Tobin, A.J., Lehmann, P.V., 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 366, 69–72.
- Keene, J.A., Forman, J., 1981. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J. Exp. Med.* 155, 768.
- Kim, J., Richter, W., Aanstoot, H.J., Shi, Y., Fu, Q., Rajotte, R., Warnock, G., Baekkeskov, S., 1993. Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. *Diabetes* 42, 1799–1808.
- Ljunggren, H.G., Stam, N.J., Ohlen, C., Neeffjes, J.J., Hoglund, P., Heemels, M.T., Bastin, J., Schumacher, T.N.M., Townsend, A.R.M., Karre, K., 1990. Empty class I molecules come out in the cold. *Nature* 346, 476–480.
- Montgomery, R.A., Dallman, M.J., 1991. Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction. *J. Immunol.* 147, 554–560.
- Namen, A.E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D.Y., Schmierer, A., Mosley, B., March, C.J., Urdal, D., Gillis, S., Cosman, D., Goodwin, R.G., 1988a. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* 333, 571–573.
- Namen, A.E., Schmierer, A.E., March, D.Y., Overell, R.W., Park, L.S., Urdal, D.L., Mochizuki, D.Y., 1988b. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J. Exp. Med.* 167, 988–1002.
- Okazaki, H., Ito, M., Sudo, T., Hattori, M., Kano, S., Katsura, Y., Minato, N., 1989. IL-7 promotes thymocyte proliferation and maintains immunocompetent thymocytes bearing  $\alpha\beta$  or  $\gamma\delta$  T-cell receptors in vitro: synergism with IL-2. *J. Immunol.* 143, 2917–2922.
- Petersen, J.S., Russel, S., Marshal, M., Kofod, H., Buschard, C.,

- Cambon, N., Karlsen, A.E., Boel, E., Hagopian, W.A., Hejnfs, K.R., Moody, A., Dyrberg, T., Lernmark, A., Madsen, O.D., Michelsen, B.K., 1993. Differential expression of glutamic acid decarboxylase in rat and human islets. *Diabetes* 42, 484–495.
- Taylor, P.M., Davey, J., Howland, K., Rothbard, J.B., Askonas, B.A., 1987. Class I MHC molecules rather than other mouse genes dictate influenza epitope recognition by cytotoxic T cells. *Immunogenetics* 26, 267–272.
- Tisch, R., Yang, X.D., Singer, S.M., Liblau, R.S., Fugger, L., McDevitt, H.O., 1993. Immune responses to glutamic acid decarboxylase correlate with insulinitis in non-obese diabetic mice. *Nature* 366 (6450), 72–75.
- Velloso, L.A., Kampe, O., Eizirik, D.L., Hallberg, A., Andersson, A., Karlson, F.A., 1993. Human autoantibodies react with glutamic acid decarboxylase antigen in human and rat but not mouse pancreas. *Diabetologia* 36, 39–46.
- Von Boehmer, H., Haas, W., 1979. Distinct Ir genes and killer cells in the cytotoxic response to H-Y antigen. *J. Exp. Med.* 150, 1134.
- Wagner, H.A., Starzinski-Powitz, A., Pfizenmaier, K., Rollinghoff, M., 1976. T-T cell collaboration during in vivo responses to antigens coded by the peripheral and central region of the MHC. *Nature* 263, 235.
- Widmann, C., Romero, P., Maryanski, J.L., Corradin, G., Valmori, D., 1992. T helper epitopes enhance the cytotoxic response of mice immunized with MHC class I-restricted malaria peptides. *J. Immunol. Methods* 155 (1), 95–99.
- Wiles, M.V., Ruiz, P., Imhof, B.A., 1992. Interleukin-7 expression during mouse thymus development. *Eur. J. Immunol.* 22, 1037–1042.
- Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, T., Streilein, J.W., Klein, J., 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T cell help. *J. Exp. Med.* 147, 897.