Immunology 1995 **84** 355–359

Major histocompatibility complex control of the class of the immune response to the hapten trinitrophenyl

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SUMMARY

This paper investigates major histocompatibility complex (MHC) regulation of the class of the immune response given *in vitro* and *in vivo* following immunization of the congenic BALB/k (H-2^k) and BALB/c (H-2^d) mice with the hapten trinitrophenyl (TNP). TNP-immune lymph node cells from BALB/k mice produced high levels of interferon-γ (IFN-γ), interleukin-5 (IL-5) and IL-2 when stimulated with TNP–antigen-presenting cells (APC) *in vitro*, while TNP-immune lymph node cells from BALB/c mice produced very low levels of these cytokines. No significant difference was found in antigen-specific production of IL-3, IL-4 and tumour necrosis factor-α (TNF-α). There was a strong correlation between the pattern of cytokine production *in vitro* and the secondary antibody production *in vivo*. Sera from BALB/k mice had anti-TNP IgG2a, IgG2b and IgG3 levels threefold greater, and anti-TNP IgA levels eightfold greater, than BALB/c mice. The level of specific IgG1 and IgE was only marginally raised in BALB/k mice. In contrast to these strain differences in cytokine and antibody production, there was no difference in two measures of cellular immunity: contact sensitivity *in vivo* and antigen-specific lymphocyte response *in vitro*. Our results suggest that there is a good correlation between the production of cytokines *in vitro* and antibody response *in vivo*, but not with measures of cellular immunity. Moreover, this MHC control of the class of the immune response to TNP does not fit into the T-helper type-1 (Th1)–Th2 paradigm.

INTRODUCTION

The recognition that the immune response (Ir) genes mapped to major histocompatibility complex (MHC) class II was the first pointer to the central role of MHC class I and class II molecules in the immune response.[1] These molecules are now known to bind peptide fragments of protein antigens, and the corresponding T-cell responses generated are specific for these MHC–peptide complexes.[2]

The role of peptide–MHC complexes in eliciting immune responses is well established, although not all immune responses have the same outcome; some are mainly humoral (antibody) responses, while others are cell-mediated immune responses.[3,4] The mechanisms that regulate the effector functions elicited in a given immune response are poorly understood, but one possibility has come from the recent definition of two different classes of $CD4^+$ helper cells among murine T-helper clones. Some cloned T cells, commonly referred to as T-helper type-1 (Th1), are specialized for cell-mediated responses and secrete the distinctive cytokines interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β). Other helper T-cell clones, commonly referred to as Th2, are specialized for inducing humoral immune responses and secrete the distinctive cytokines IL-4, IL-5, IL-6 and IL-10.[5] However, during *in vivo* immune responses to most antigens, CD4 T cells of both types appear to be activated in that both antigen-specific T-cell proliferation and T-cell help have been observed.[6]

We have found previously that the production of IFN-γ and IL-5, following immunization with the hapten trinitrophenyl (TNP) and restimulation of immune lymph node cells with specific antigen *in vitro*, is controlled by MHC. In fact, using congenic strains of mice that differ in MHC genotype, it was found that BALB/k mice produced both IFN-γ and IL-5, while the congenic BALB/c mice virtually failed to produce appreciable amounts of these cytokines.[7,8]

The observation that a single, simple chemical hapten (TNP) introduced in the same form and by the same route of immunization was able to generate different classes of immune responses based on the different MHC genotypes opened the possibility that MHC regulates which CD4 T-cell subset is activated by a given antigen.[9] Therefore, we extended our studies of MHC control of the class of the immune response to

Received 10 August 1994; revised 5 November 1994; accepted 13 November 1994.

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TNP, analysing the pattern of cytokine production in response to TNP, as well as antigen-specific humoral and cell-mediated immune responses *in vivo* in BALB/k and BALB/c mice.

MATERIALS AND METHODS

Mice

BALB/k (H-2^k) and BALB/c (H-2^d) male mice were purchased from Olac Ltd (Bicester, UK) and were bred at the Institute of General Pathology (University of Palermo, Italy). The mice were used at an age of 8–12 weeks and each experimental group contained at least six mice.

Sensitization with TNP

Mice were painted on the shaved thorax and abdomen with 0·2 ml of 5% pichryl chloryde (TNP; BDH, Poole, UK) dissolved in a 1 : 3 ethanol : acetone mixture.

Contact sensitivity (CS) reaction

Mice were painted with TNP as described above. Seven days later the mice were challenged on both sides of both ears with 1% TNP dissolved in olive oil, and the increase in ear swelling was measured after 24 hr with an engineer's micrometer and expressed in units of 10^{-3} cm ± standard deviation (SD).

Serum antibody assay

Total and anti-TNP serum antibodies were measured using an enzyme-linked immunosorbent assay (ELISA). Total serum antibodies were analysed using commercially available reagents (Sigma, St Louis, MO).

To assay anti-TNP serum antibodies, mice were painted on day 0 with TNP. On day 14 the mice were boosted by painting with 3% TNP, and they were bled on day 25.[10,11]

Plastic microtitre plates (Nunc Immunoplate type II; Nunc, Copenhagen, Denmark) were coated with 100 μl of 100 μg/ml of TNP–bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7·2, by overnight incubation at 4°. After two washes with PBS, sera were incubated in a volume of 100 μl/well for 2 hr at 4°. The plates were washed twice with PBS–Tween and goat anti-mouse immunoglobulin isotypes (Sigma) were added, after dilution as recommended by the manufacturer. After 60 min of incubation at room temperature, the plates were washed twice with PBS–Tween and 100 μl of peroxidase-labelled rabbit anti-goat immunoglobulin (Sigma) was added in each well. After washing, the plates were incubated with H_2O_2 and o-phenylendiamine. The reaction was stopped after 15–30 min by addition of 0·5 M citric acid.

Substrate conversion was measured using an automatic ELISA reader. Antibody concentration was expressed as the mean optical density (OD) at 492 nm of triplicate wells, corrected for background staining recorded with normal mouse serum at an equivalent dilution. Values were considered positive when the OD value obtained by diluted immune sera was greater than the mean value ± 2 SD of diluted control sera. For all anti-TNP immunoglobulin isotypes, levels were calculated by using a standard curve generated with monoclonal anti-TNP mouse antibodies of specific isotypes. However, because *in vivo* responses are polyclonal and reflect antibodies of varying affinities for the antigen, expressing the anti-TNP immunoglobulin in concentrations unit equivalent to anti-TNP monoclonal standards would only be an approximate comparison. Therefore, the antibody titres were plotted as OD 492 nm readings.

Proliferation assay

Mice were immunized with TNP as described above. Four days later, draining lymph node cells were harvested, pooled and washed three times in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with streptomycin, penicillin, glutamine, sodium pyruvate, 2-mercaptoethanol (2×10^{-5} M) and 10% heat-inactivated fetal calf serum. 4×10^5 cells in 100 μl of medium were cultured in flat-bottomed 96-microtitre plates with irradiated (3000 rads from a caesium source), TNP-modified syngeneic spleen cells (prepared as described elsewhere)[7] as antigen-presenting cells (APC) (4×10^5 in 100 μl). One μCi of methyl-^{3}H-thymidine ([^{3}]TdR; Amersham Int., Amersham, UK) was added to each well 48 hr later, and the following day the culture was harvested with a multichannel cell harvester (Skatron, Lier, Norway). Results are expressed as mean counts per minute (c.p.m.) of triplicate cultures ± SD.

Lymphokine production and assay

Mice were immunized with TNP, and draining lymph node cells from all the mice in a group were harvested, pooled and washed as described above. Culture supernatants were obtained by incubating 4-day TNP-immune lymph node cells (10^7) with TNP–APC (10^7) in a total volume of 2 ml. Alternatively, 4-day TNP-immune lymph node cells (10^7) were stimulated with concanavalin A (Con A; 5 μg/ml final concentration) in a total volume of 2 ml. After 24 hr of incubation at 37° in the presence of 5% CO_2, supernatants were collected by centrifugation and filtration and stored at −70°.

IFN-γ was tested by its ability to inhibit the cytopathic effect of encephalomyocarditis (EMCV) virus on L929 cells.[7] TNF-α activity was tested by a commercially available ELISA assay (Genzyme). IL-2 and IL-4 were tested using CTLL cells in the presence of the anti-IL-4 mAb 11B11 and anti-IL-2R 7D4.[12] IL-3 activity in supernatants was tested using DA1 cells.[12] IL-5 was detected by a costimulatory assay based on the ability of IL-5 to promote and support growth of murine B cells exposed to submitogenic doses of dextran sulphate.[11] Specificity of the biological assays was confirmed by inhibition with the neutralizing monoclonal antibodies R4-6A2 (anti-IFN-γ), MP2-8F8 (anti-IL-3) and TRFK5 (anti-IL-5) (Pharmingen, San Diego, CA).

Statistics

The double Student's *t*-test was used to evaluate the significance of experimental versus control group data.

RESULTS

Production of lymphokines by 4-day immune lymph node cells from BALB/c and BALB/k mice

Previous papers from our laboratories have demonstrated that the production of IFN-γ and IL-5, following immunization *in vivo* with TNP and restimulation *in vitro* with specific antigen, is controlled by the MHC in the congenic BALB/c and BALB/k mice.[7,8] We investigated whether MHC regulation of cytokine production affected cytokines other than IFN-γ and IL-5. For this purpose, TNP-immune lymph node cells from BALB/k and BALB/c mice were re-exposed *in vitro* to TNP-modified spleen

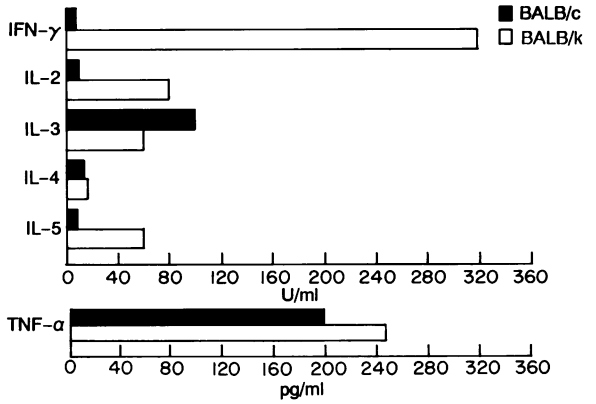

Figure 1. Pattern of cytokine production by 4-day TNP immune lymph node cells from BALB/k and BALB/c mice, re-exposed to antigen *in vitro*. Cytokine levels in lymph node cells from TNP-immune mice stimulated with haptenized (TNP) syngeneic APC are shown. Lymph node cells from TNP-immune mice incubated in medium virtually produced no detectable cytokine. Haptenized (TNP) syngeneic APC incubated in medium produced only low levels of TNF-α (10 pg/ml) and IL-3 (10 U/ml). Results are expressed in U/ml or pg/ml (TNF-α) ± SD.

cells, and culture supernatants were harvested 24 hr later and tested for the presence of several cytokines. Figure 1 shows that BALB/k immune lymph node cells produced IFN-γ, IL-2, IL-3, IL-4, IL-5 and TNF-α when stimulated with TNP *in vitro*. In contrast, BALB/c immune lymph node cells, although producing IL-3, IL-4 and TNF-α in similar amounts to BALB/k cells, produced very low levels of IFN-γ, IL-2 and IL-5. Similar amounts of cytokine were produced by 4-day immune lymph node cells from BALB/c and BALB/k mice stimulated by Con A *in vitro* (Fig. 2). Identical results were obtained in three different experiments. We therefore concluded that MHC control of cytokine production in response to TNP involves at least three cytokines, namely IFN-γ, IL-2 and IL-5.

TNP-specific secondary antibody response in BALB/c and BALB/k mice

It is known that cytokines modulate several aspects of the

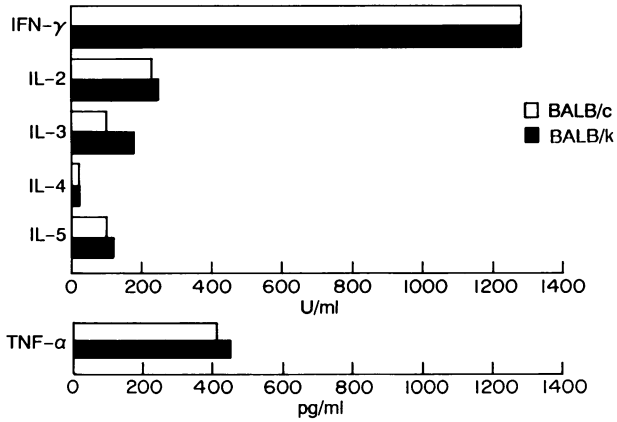

Figure 2. Pattern of cytokine production by 4-day TNP immune lymph node cells from BALB/k and BALB/c mice, stimulated with Con A *in vitro*. See legend to Fig. 1 for further details.

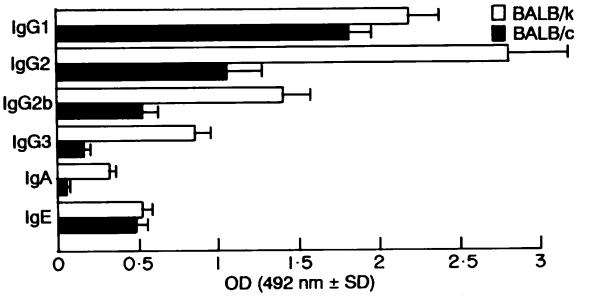

Figure 3. Antigen (TNP)-specific antibody levels in sera from BALB/k and BALB/c mice. Results are expressed as mean OD value at 492 nm ± SD. The following OD value differences between BALB/c and BALB/k mice reached statistical significance: IgG2a ($P < 0{\cdot}005$), IgG2b ($P < 0{\cdot}005$), IgG3 ($P < 0{\cdot}005$) and IgA ($P < 0{\cdot}001$).

immune response *in vivo*. For instance, certain cytokines exert synergistic or antagonistic effects on heavy chain class switching in murine B lymphocytes.[13] We therefore asked whether the different pattern of cytokine production *in vitro* by BALB/k and BALB/c influenced the *in vivo* anti-TNP serum antibody response.

Figure 3 shows that sera from BALB/k mice had anti-TNP, IgG2a, IgG2b and IgG3 levels threefold greater, and anti-TNP IgA levels eightfold greater, than sera from BALB/c mice, in keeping with our previous results. The BALB/k mice had somewhat higher anti-TNP IgG1 levels which were not significantly different from BALB/c mice, while the anti-TNP IgE levels were somewhat higher in BALB/k than in BALB/c mice, but the difference did not reach significance.

The levels of total immunoglobulin isotypes did not differ in sera from BALB/c and BALB/k mice (data not shown).

Cell-mediated immune response to TNP in BALB/c and BALB/k mice

Two different aspects of the cell-mediated immune response to TNP were analysed in BALB/c and BALB/k mice, namely the CS reaction *in vivo* and antigen-specific lymph node cell proliferation *in vitro*. Figure 4 shows the result of a typical experiment of CS. The mean ear swelling to challenge on day 7

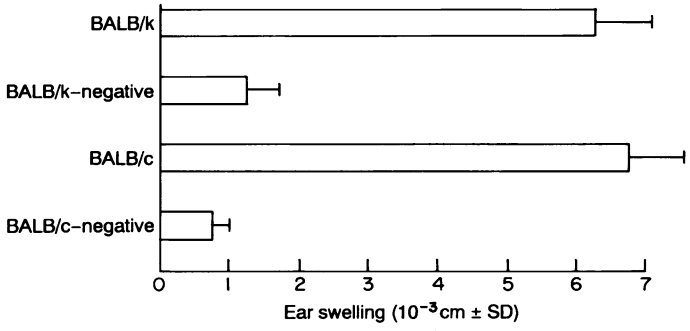

Figure 4. CS reaction to TNP in BALB/k and BALB/c mice. BALB/k and BALB/c mice were immunized with TNP and challenged with TNP 7 days later (BALB/k and BALB/c groups). BALB/k- and BALB/c-negative groups refer to mice not immunized with TNP (negative control groups). The ear swelling value differences between the BALB/k and the BALB/c group did not obtain statistical significance.

Table 1. Antigen-specific proliferative response by TNP-immune lymph node cells for BALB/c and BALB/k mice

'Day 4' TNP-immune lymph node cells	Antigen (TNP–spleen cells)	Thymidine incorporation (c.p.m.) + SD)
BALB/k	—	4605 ± 670
BALB/k	BALB/k	13934 ± 890
BALB/c	—	3170 ± 1090
BALB/c	BALB/c	12099 ± 1233

Irradiated BALB/k APC yielded 540 ± 120 c.p.m. and irradiated BALB/c APC yielded 600 ± 320 c.p.m.

was virtually the same in BALB/k and BALB/c mice. Moreover, the proliferative response of the 4-day TNP-immune lymph node cells to TNP-modified spleen cells *in vitro* was similar. Table 1 reports the data from the proliferation assay and shows that BALB/k and BALB/c 4-day lymph node cells proliferated to the same extent in response to the specific antigen *in vitro*.

DISCUSSION

The present investigation was prompted by previous results from our laboratories showing MHC control of certain classes (types) of immune responses to simple chemical haptens in the mouse. This was formally demonstrated using congenic BALB/c and BALB/k mice. In fact, TNP-immune lymph node cells from BALB/k mice produced high levels of IFN-γ and IL-5 when stimulated with specific antigen *in vitro*, while TNP-immune lymph node cells from BALB/c mice produced very low levels of these cytokines under the same experimental conditions.

This paper extends the phenomenon of MHC control of the class of the immune responses to the analysis of cytokines other than IL-5 and IFN-γ, furthermore, it investigates whether the different pattern of cytokine production following immunization of BALB/c and BALB/k mice with TNP and re-exposure to antigen *in vitro* is correlated with antigen-specific humoral and cell-mediated responses *in vivo*. In fact, it has already been reported that epicutaneous immunization with haptens leads to the activation of both cellular and humoral immunity, both phenomena having a role for T-cell derived cytokines.[12]

Initial experiments showed that not only the production of IFN-γ and IL-5, but also the production of IL-2, was controlled by MHC. Data reported in Fig. 1 show that TNP-immune lymph node cells from BALB/k mice produced high levels of IFN-γ, IL-5 and IL-2 when stimulated with TNP–APC *in vitro*, while TNP-immune lymph node cells from BALB/c mice produced very low levels of these cytokines. No difference was found in antigen-specific production of IL-3, IL-4 and TNF-α. Our results confirm the production of IL-4 following immunization with TNP, as detected either by polymerase chain reaction (PCR) amplification of cDNA or a sensitive biological assay.[14]

As IFN-γ is regarded as an important mediator of the CS reaction,[15] the finding that BALB/c mice, although producing very low levels of IFN-γ, show a normal CS reaction *in vivo* merits some comments. In fact, published reports differ on the role of IFN-γ in the CS reaction. Anti-IFN-γ antibody has been reported to cause no inhibition,[16] or enhancement[17] of the CS reaction. These observations suggest that IFN-γ is not solely for the CS reaction, and an important role may be played by IL-3[12,16] and TNF-α[18] which are normally produced by BALB/c mice. Similarly, the observation that BALB/c mice show a normal antigen-specific proliferation *in vitro* but produce low levels of IL-2 is not surprising, in that very low amounts of IL-2, below the lower limit of the biological assay, can support proliferation, as described in the mixed lymphocyte reaction in the mouse.[19] This conclusion is supported by results from Janeway *et al.*[20] in the MHC-controlled response to human collagen type IV. In fact, I-A^s and I-A^d mice made comparable amounts of IL-2 *in vitro* but only cells from I-A^s mice were able to mount a proliferative response *in vitro*, which suggests that IL-2 receptor (R) expression, rather than IL-2 production, is a marker for T-cell proliferation.

The key finding was the strong correlation found between the pattern of cytokine production *in vitro* and secondary antibody response *in vivo*, in BALB/c and BALB/k mice. Sera from the high cytokine producer strain BALB/k had anti-TNP IgG2a, IgG2b and IgG3 levels threefold higher, and anti-TNP IgA levels eightfold higher, than in BALB/c mice. With regard to antibody production, the raised levels of IgG2a, IgG2b and IgG3 in the high cytokine producer strain were compatible with the key role of IFN-γ in their production, while the raised levels of IgA might reflect the increased production of IL-5. The comparable level of IgG1 and IgE in BALB/k and BALB/c mice, which are strongly influenced by IL-4, might be due to the production of comparable amounts of IL-4.

It has been reported that IFN-γ antagonizes the effect of IL-4 on B-cell growth and isotype expression, in that it induces switching to IgG2a and inhibits expression of IgG1 and IgE.[21] Our results show that, in BALB/k mice, the high IFN-γ production, although inducing high anti-TNP IgG2a levels, does not block either IL-4 production or IL-4-dependent anti-TNP IgG1 and IgE expression. This suggests that IFN-γ, although able to positively regulate induction of certain immunoglobulin isotypes (IgG2a), fails to negatively regulate production of IL-4-induced immunoglobulin isotypes (IgG1 and IgE).

Our results are in full agreement with the results obtained by Thomson *et al.*,[16] who demonstrated that monoclonal antibodies against IFN-γ were able *in vivo* to reduce anti-oxazolone IgG2a levels while failing to affect anti-oxazolone IgG1 levels. On the other hand, IL-4 transgenic mice showed an increased production of anti-TNP IgG1 and IgE, only the latter being inhibited by anti-IL-4 monoclonal antibody.[22] However, our results leave open the question of why BALB/k mice produce as much anti-TNP IgE as BALB/c mice while producing much more IFN-γ, but one possibility may be that the enhancing effect of IL-4 on IgE synthesis is 'dominant' over the IFN-γ inhibitory effect, perhaps because the IL-4-dependent switching to IgE requires very low amounts of IL-4 and its inhibition requires very high amounts of IFN-γ.

In conclusion, our results clearly show that MHC plays a critical role in the control of the class of the immune response either *in vivo* or *in vitro*, and there is a good correlation between the pattern of cytokines produced *in vitro* and the antibody response *in vivo*. However, there is no correlation with two measures of cellular immunity, CS reaction and antigen-driven

lymph node cell proliferation *in vitro*. Furthermore, the finding that immunization with TNP induces both humoral and cell-mediated immune responses and a complex pattern of cytokine production indicates that this response is not predominantly of either Th1 or Th2 type.

ACKNOWLEDGMENTS

This work has been supported by grants from the Italian National Research Council (CNR, Rome) and from the Ministry for University and Scientific and Technological Research (MURST 40% and 60%). We would like to thank Professor N. A. Mitchison for reviewing the manuscript and for indirect contribution to this work.

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