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In vitro studies on H-2 linked unresponsiveness

1. NORMAL HELPER CELLS TO (T,G)-A–L AND GAT IN LOW AND NON-RESPONDER MICE

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Summary. Lymphoid cells from unprimed high responder (C57BL/10) and low responder mice (B10.Br, B10.A, CBA) to (T,G)-A–L and high responder (B10, B10.A) and non-responder (B10.G, DBA/I) mice to GAT can be induced to form antigen specific T-helper cells *in vitro* under identical culture conditions. The helper cells induced from high and low or non-responder mice appear to be identical in efficiency, antigen concentration requirement for induction and induction kinetics.

INTRODUCTION

The immune response to synthetic polypeptides such as poly-L (Tyr, Glu)-poly-DL-Ala–poly-L-Lys, abbreviated to (T,G)-A–L, and the random terpolymer of 60 per cent L-glutamic acid, 30 per cent L-alanine and 10 per cent L-tyrosine, abbreviated GAT^{10}, have been shown to be under the control of dominant immune response genes which are localized within the major histocompatibility complex (MHC), H-2 in the mouse (reviewed by Benacerraf & Katz, 1975). Similar MHC-linked genetic restrictions in responsiveness have been found in other species such as the guinea-pig (Ellman, Green Martin & Benacerraf, 1970), the rat (Gunther, Rude & Stark, 1972), and the Rhesus monkey (Dorf, Balner, de Groot & Benacerraf, 1974).

Over the last few years much work has been performed to attempt to trace the site of action of H-2-linked unresponsiveness. Some experiments have pointed to a defect of T-cell function (McDevitt, 1968; particularly of T-cell receptors (Benacerraf & McDevitt, 1972; Hammerling, Masuda & McDevitt, 1973), while others have suggested defects in B-cell function (Mozes & Shearer, 1971) or both B- and T-cell defects (Shearer, Mozes & Sela, 1972; Lichtenberg, Mozes, Shearer & Sela, 1974; Mozes, Isac & Taussig, 1975; Mozes, Shearer, Maron, Arnon & Sela, 1973; Munro & Taussig, 1975); other hypotheses have been the induction of B-cell tolerance (Kapp, Pierce & Benacerraf, 1974) or of suppressor T cells in low or non-responder mice (Gershon, Maurer & Merryman, 1973; Kapp *et al.*, 1974).

Since the immune responses to (T,G)-A–L and GAT^{10} have been extensively studied, chiefly *in vivo* reviewed by Benacerraf & Katz (1975), they were chosen for this *in vitro* study of the function of the various cellular components (T cells, B cells and macrophages) in H-2-linked immune responses. *In vitro* techniques were chosen because of the

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recently developed techniques for the induction of helper cells (Kontiainen & Feldmann, 1973) and suppressor cells (Kontiainen & Feldmann, 1976) *in vitro* and because of the ease of studying both the function of each cell type involved (Erb & Feldmann, 1975a) and of various T-cell factors (Feldmann & Basten, 1972). Furthermore, these techniques enable the function of both primed and unprimed T and B cells to be studied, the latter being difficult *in vivo*.

This communication describes the normal helper cell response of low responder T cells of the B10.A and B10.Br strains to (T,G)-A–L, and of non-responder T cells of the DBA/1 and B10.G strains to GAT ^{10} *in vitro*.

MATERIALS AND METHODS

Animals

Mice congenic with C57 Bl/10 (B10, H-2^{b})-B10.Br (H-2^{k}), B10.A (H-2^{a} or k/d) and B10.G (H-2^{q}), and DBA/1 (H-2^{q}) mice were either kindly donated by Dr E. Simpson of the Clinical Research Centre, Harrow, or bred at University College London. Usually, mice were used at 3–6 months of age, at least 1 month after vaccination against ectromelia.

Antigens

Poly-L-(Tyr, Glu)-poly-DL-Ala–poly-L-Lys, (T,G)-A–L, batch no. 1383 was prepared as described by Sela, Fuchs & Arnon (1962). The random terpolymer of 60 per cent glutamic acid, 30 per cent alanine, 10 per cent tyrosine, GAT^{10}, was prepared as described by Katchalski & Sela (1958) and had a mol. wt of 50,000 daltons. Dinitrophenylated (T,G)-A–L, DNP-(T,G)-A–L was prepared as described by Sela & Mozes (1966). Conjugates had approximately four groups of DNP per 150,000 daltons of (T,G)-A–L.

4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was conjugated onto GAT^{10} to form NIP-GAT, by the low-coupling ratio method of Brownstone, Mitchison & Pitt-Rivers (1966). Conjugates had approximately two groups of NIP per molecule of GAT.

Antisera

(a) *Anti-T cell serum.* A sheep anti-mouse thymocyte serum (anti-T) was prepared by Dr I. McConnell, absorbed with mouse erythrocytes and tested against ^{51}Cr-labelled mouse spleen cells and thymocytes in a cytotoxicity assay to determine its activity. Appropriately absorbed serum lysed 90 per cent of thymocytes and 45 per cent of spleen cells at a dilution of 1/250 with no detectable effect on B cells.

(b) *Anti-B cell serum.* A rabbit anti-mouse B-cell serum was prepared as described previously (Erb & Feldmann, 1975a), absorbed with mouse thymocytes and erythrocytes and tested against ^{51}Cr-labelled mouse spleen cells and thymocytes in a cytotoxicity assay to determine its activity. Appropriately absorbed serum lysed 45 per cent of spleen cells, but no thymocytes, at a final dilution of 1/100. After anti-B treatment residual B cells ranged from 0–2 per cent, using fluorescent anti-Ig as a marker of B cells.

Purification of spleen cells

Absorbed anti-B serum, in the presence of spleen cell-absorbed guinea-pig complement was used to deplete spleen cell suspensions of B cells. Carbonyl iron treatment was also used, as described elsewhere (Erb & Feldmann, 1975b). This procedure leads to a significant but variable loss of B cells.

Purified populations of B cells were obtained by treating spleen cells with anti-T serum and guinea-pig complement for 1 h at 37°; residual T cells varied from 0–5 per cent as judged by lysis with anti-θ serum.

Populations of spleen cells were depleted of macrophages by carbonyl iron treatment. Macrophage depleted populations were reconstituted by the addition of 3 per cent peritoneal exudate cells from normal mice, or from mice which had been stimulated by the injection of 2 ml of 2 per cent starch solution i.p. 48–72 h previously.

Tissue cultures

(a) *Helper cell induction.* Spleen or peripheral lymph node cells were removed and teased into single cell suspensions in HEPES-buffered Eagles minimal essential medium (HEM) containing 10 per cent foetal calf serum (FCS). The cells were washed through FCS, resuspended in HEM containing 10 per cent FCS and their viability determined by trypan blue exclusion. 15×10^{6} viable cells, mixed with an appropriate concentration of (T,G)-A–L or GAT were diluted to 1 ml in HEM containing 10 per cent FCS and placed in the inner chamber of a Diener-Marbrook tissue culture flask. Approximately 25 ml of bicarbonate-buffered MEM containing 5 per cent calf serum and 5×10^{-5} M of

2-mercaptoethanol (2ME–MEM) was placed in the outer chamber of each flask. Triplicate cultures were then incubated for 4 days at 37° in a constant humidity CO_2 incubator. After 4 days the cells were harvested, washed through FCS, resuspended in HEM containing 10 per cent FCS, and their viability determined using typan blue. The viability (per cent survival from original culture) ranged from 15–35 per cent. These cells were then used as helper cells (HC) in the subsequent cooperative culture.

(b) *Co-operative culture.* 15×10^6 fresh syngeneic spleen cells, together with an appropriate number of viable helper cells and an appropriate dose (1 μg/l ml) of DNP-(T,G)-A–L or NIP-GAT were diluted up to 1 ml in HEM containing 10 per cent FCS and placed in the inner chamber of a Diener–Marbrook culture flask. Approximately 25 ml of 2ME–MEM was placed in the outer chamber of the flask. Triplicate cultures were then incubated as above for 3 days.

After 3 days the cultures were harvested and the numbers of IgM anti-DNP or anti-NIP antibody forming cells in each culture determined in Cunningham assays, using sheep erythrocytes coated with DNP or NIP-Fab (Strausbach, Sulica & Givol, 1970).

RESULTS

Although results are only given for B10.A low responder (LR) mice to (T,G)-A–L and B10.G non-responder (NR) mice to GAT, comparable results were also obtained with B10.BR LR mice to (T,G)-A–L and the non-congenic DBA/1 NR mice to GAT^{10}.

Each graph represents data from one experiment, but each experiment has been repeated at least five times. Each data point represents the mean ± s.e. of three cultures.

Concentration of antigen for helper cell induction

To determine whether HR and LR or NR spleen and lymph node cells could be induced to form T-helper cells in vitro, 15×10^6 cells were cultured with varying concentrations of (T,G)-A–L or GAT^{10} for 4 days. At the end of the culture period triplicate cultures were harvested, washed, resuspended and their per cent viability determined by trypan blue exclusion.

10^5 viable cells were then added to 15×10^6 fresh syngeneic spleen cells together with 1 μg/ml DNP-(T,G)-A–L or NIP-GAT and recultured. After 3 days triplicate cultures were harvested and the numbers of IgM anti-DNP or -NIP plaque-forming cells per culture determined in a Cunningham assay. (No IgG plaques were detected.)

The results of these experiments are shown in Fig. 1a and b.

(a) *(T,G)-A–L helper cells*
Both HR and LR spleen and lymph node cells produce helper cells on *in vitro* incubation with antigen. Optimal spleen HC are obtained with 1 μg/ml (T,G)-A–L, and lymph node HC when grown with 10 μg/ml. However in both cases the antigen concentration optima are the same for both HR and LR cells.

(b) *GAT^{10} helper cells*
Similarly, HR and NR spleen and lymph node cells contain helper cell precursors to GAT^{10} and the antigen concentration optima are in the same range (1 μg/ml for HR and NR spleen, 10 μg/ml for HR lymph node and 5 μg/ml for NR lymph node).

Time for optimal helper cell induction

It was thought possible that HR helper cells would appear at a different time from LR or NR helper cells, so the kinetics of helper cell induction for HR and LR or NR cells were compared.

Triplicate helper-cell cultures were incubated with antigen using previously determined optimal doses (see above) for 3, 4 or 5 days, harvested, counted and added with antigen to syngeneic spleen cells in co-operative culture for 3 days. Triplicate cultures were then harvested and the numbers of IgM antibody forming cells per culture determined. The results of these experiments can be seen in Fig. 2. No significant differences were noted between HR and LR or NR cells. The optimum incubation time for helper cell induction is 4 days for both (T,G)-A–L and GAT^{10}.

Helper cell efficiency

A possible explanation for unresponsiveness could be that LR or NR helper cells are less efficient than those of HR mice. We tested this by performing a series of limiting dilutions of helper cells in 15×10^6 syngeneic spleen cells.

Helper cells induced in the optimal way (4 days in the presence of 1 μg/ml for spleen cells or 10 μg/ml for lymph node cells) were added in varying numbers (in the range 10^3–10^6) to co-operative cultures of spleen cells containing DNP-(T,G),-A-L or NIP-GAT. The results can be seen in Figs 3a and b.

(a) *(T,G)-A-L*
The optimal helper cell number lies between 10^5 and 2×10^5 for HR and LR spleen and lymph node cells. HR helper cells are not more efficient than the LR helper cells tested (B10.A or B10.Br).

(b) *GAT*
The optimal helper cell number lies between 5×10^4 and 2×10^5 for HR and NR splenic helper cells. As with (T,G)-A-L HR helper cells are not more efficient than the NR cells tested (B10.G or DBA/1).

Cellular requirements for helper-cell induction
With antigens not under genetic control, such as

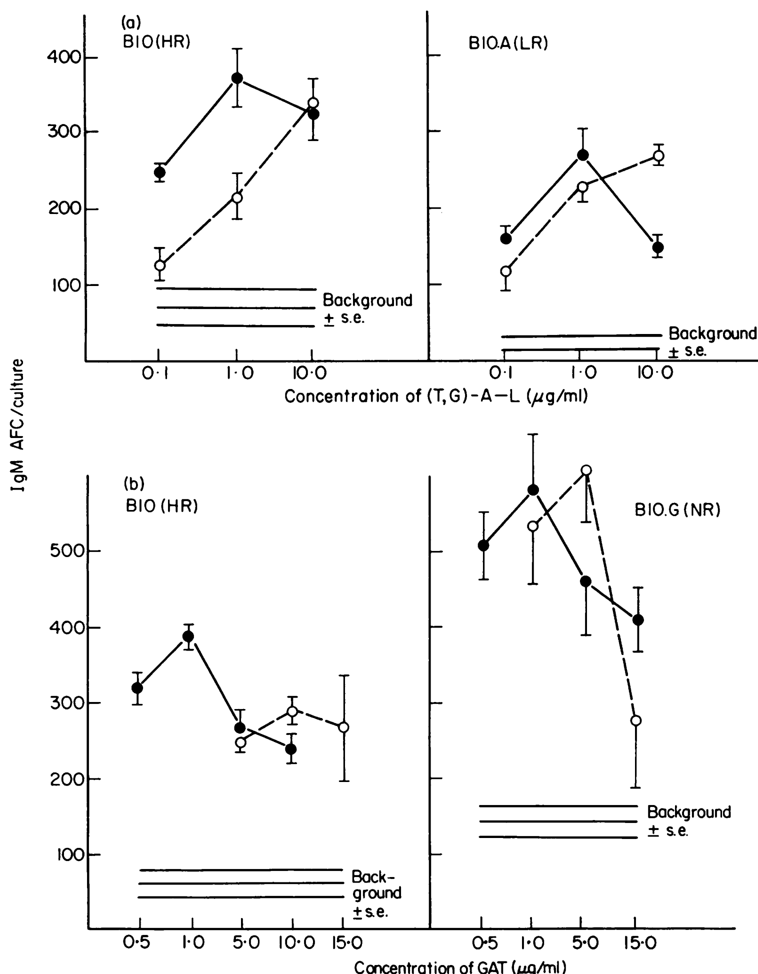

Figure 1. Optimal antigen concentration for helper-cell induction in lymph node (– – – –) and spleen cells (——). For both (T,G)-A-L (a) and GAT^{10} (b) the optimum concentration of antigen required for helper cell induction in high responder (HR) and low responder (LR) or non responder (NR) is the same. As previously described for KLH (Erb & Feldmann 1976b), lymph node cells require a higher dose than spleen cells for both (T,G,)-A-L and GAT^{10} in all mouse strains tested. Helper cells from HR and LR or NR strains induce similar numbers of IgM antibody priming cells (AFC) when added to 15×10^6 syngeneic spleen cells and haptenated antigen in cooperative culture.

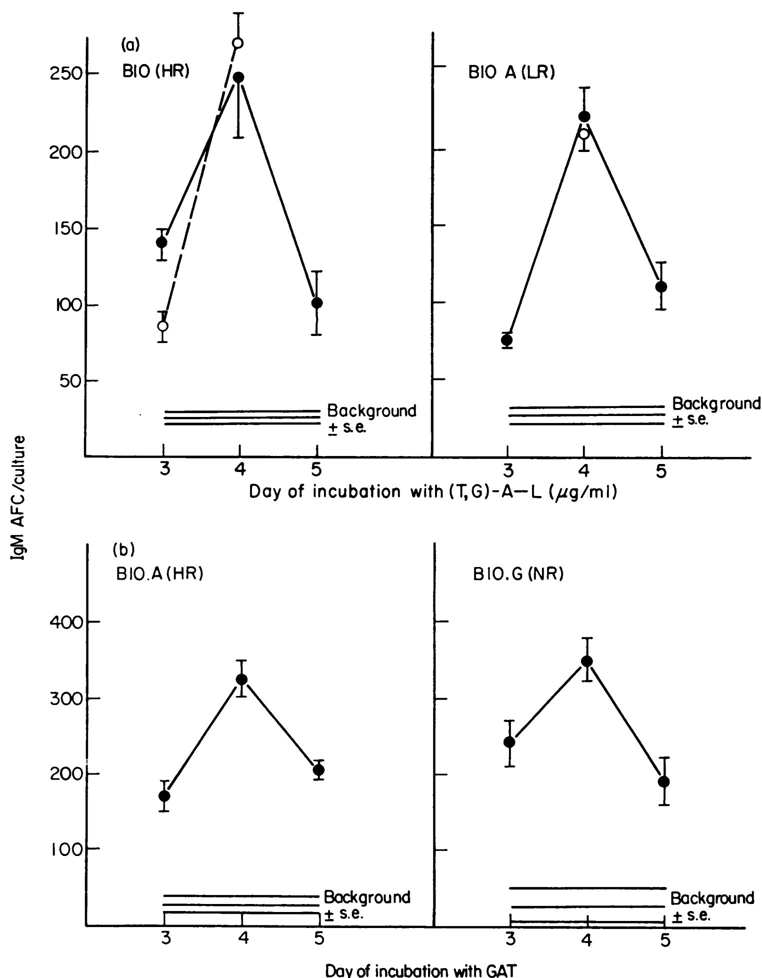

Figure 2. Kinetics of helper-cell induction for both (T,G,)-A–L (a) and GAT[10] (b). The optimal time of incubation with antigen for helper-cell induction is 4 days for HR and LR or NR cells. There are no kinetic differences in induction between HR and LR or NR cells. (——) spleen; (– – –) lymph node.

KLH, HC induction requires the cooperation of T cells and macrophages (Erb & Feldmann, 1975a and b), but not of B cells. It was of interest to verify that this was the same with antigens under Ir gene control.

Spleen cells were purified into either T- or B-cell enriched populations by treatment with anti-T or anti-B cell serum in the presence of guinea-pig complement. They were depleted of macrophages by treatment with carbonyl iron and macrophage depleted populations were reconstituted by the addition of a per cent peritoneal exudate macrophages. Helper cell cultures were set up in the usual way i.e. 4 days incubation of 15×10^6 cells and 1 μg (T,G)-A–L per ml. The results of these experiments with (T,G)-A–L are shown in Fig. 4.

The results were the same for both HR and LR cells. The anti-B treated cells showed a slight, but not significant, enhancement of helper activity, the anti-B and macrophage depleted populations showed very little helper activity, and the macrophage depleted and reconstituted populations showed normal helper activity, as compared with helper cells derived from untreated spleen cells. These results are analogous to those found with other antigens (Erb & Feldmann, 1975a and b, Kontiainen & Feldmann, 1973).

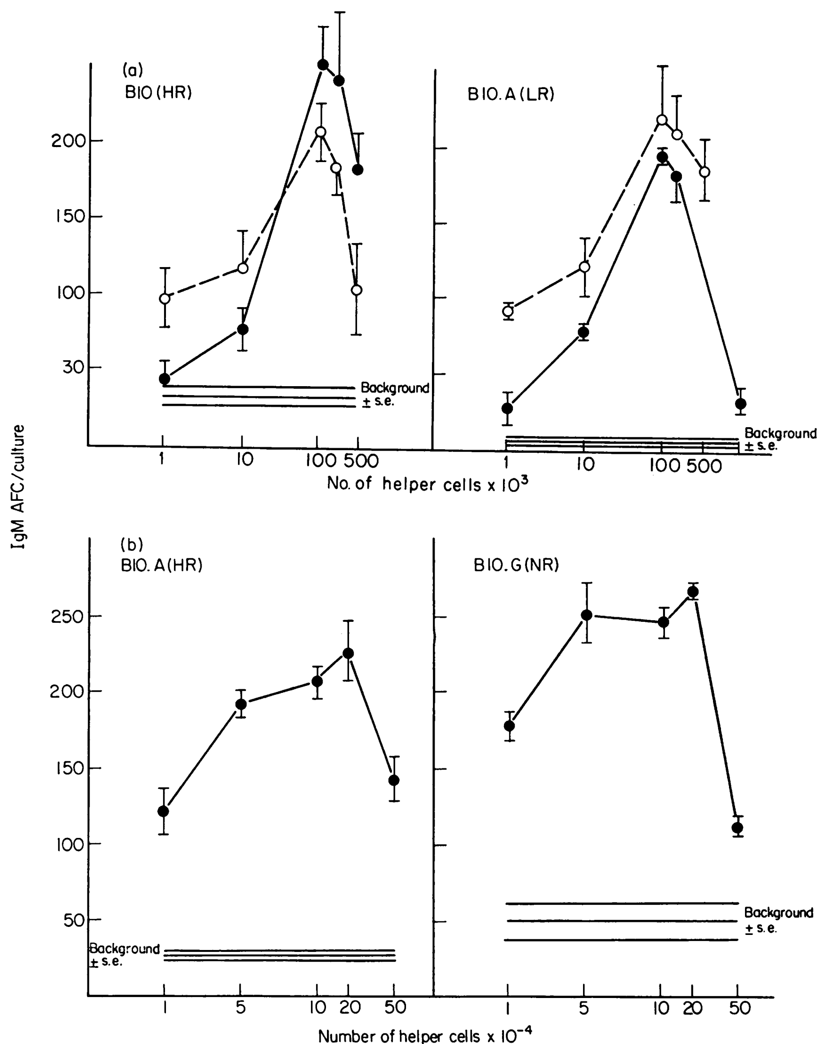

Figure 3. Helper-cell efficiency for both (T,G)-A–L (a) and GAT[10] (b). The optimal number of helper cells added to 15×10^6 syngeneic spleen cells and haptenated antigen in co-operative culture is identical in the HR and LR or NR strains tested. Low or non-responder helper cells are just as efficient at inducing IgM-antibody-forming cells (AFC) as high responder helper cells. (——) spleen; (– – –) lymph node.

Antigenic specificity of helper cells

To show that the helper cells were antigen specific (T,G)-A–L spleen helper cells were added to syngeneic spleen cells together with NIP–GAT and the numbers of anti-NIP AFC precursors determined; similarly GAT spleen helper cells were added to syngeneic spleen cells together with DNP-(T,G)-A–L and the numbers of anti-DNP AFC per culture were determined. The results can be seen in Table 1. Helper cells induced with GAT[10] will only help when NIP-GAT is used as antigen, not when DNP-(T,G)-A–L is supplied; helper cells induced with (T,G,)-A–L will only help when DNP-(T,G)-A–L is used as antigen and not when NIP-GAT is used. If both HC types and both antigens are cultured with 15×10^6 spleen cells both anti-NIP and anti-DNP PFC are found. The helper cells are therefore antigen specific and do not compete with each other in the experimental system described.

These results also indicate that T-cell specificity

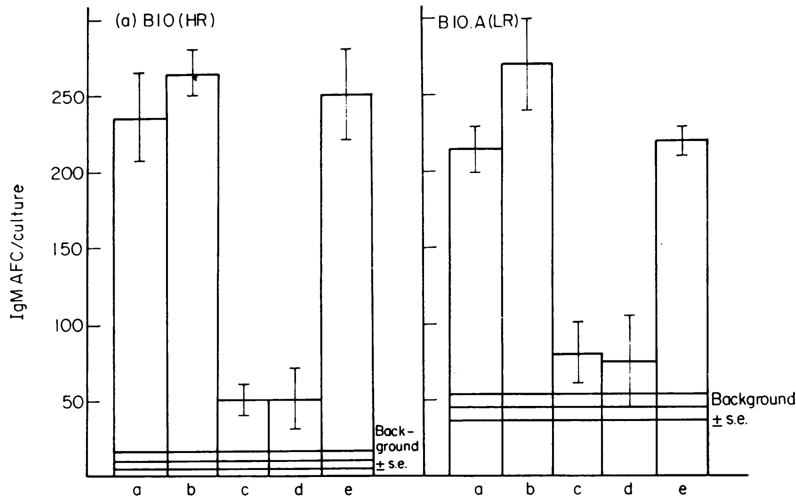

Figure 4. Cellular requirements for (T,G)-A–L helper cell induction. Helper cells derived from: (a) whole spleen; (b) anti-B cell and complement-treated spleen; (c) anti-T cell and complement-treated spleen; (d) macrophage-depleted spleen; (e) macrophage-depleted and reconstituted spleen. In both high and low responder cells to (T,G)-A–L the cellular requirements for T-helper cell induction are the same.

Table 1. Antigen specificity of helper cells to GAT^{10} and (T,G)-A–L

Helper cells	Cells cultured spleen cells	Antigen	Response (AFC/cultures ± s.e.) Anti-DNP	Anti-NIP
—	B10	DNP (T,G)-A–L	0	—
^{HC}(T,G)-A–L	B10	DNP (T,G)-A–L	187 ± 7	—
$^{HC}GAT^{10}$	B10	DNP (T,G)-A–L	20 ± 6	—
—	B10	NIP GAT	—	133 ± 13
$^{HC}GAT^{10}$	B10	NIP GAT	—	347 ± 17
^{HC}(T,G)-A–L	B10	NIP GAT	—	83 ± 7
^{HC}(T,G)-A–L + $^{HC}GAT^{10}$	B10	NIP GAT + DNP (T,G)-A–L	223 ± 12	253 ± 9

Cross reactivity experiments were done using helper/spleen cells induced to (T,G)-A–L and GAT^{10} in B10 mice (HR to both antigens). The helper cells are non cross-reactive, indicating that T-helper cells (or factors produced by them) have different specificities from the antibodies produced against (T,G)-A–L and GAT^{10} which are cross reactive.

for antigen is different from that of antibody since antibodies to (T,G)-A–L and GAT are cross-reactive (Maurer, unpublished work).

DISCUSSION

This communication describes the *in vitro* induction of helper cells to two different synthetic polypeptide antigens, (T,G)-A–L and GAT^{10}, using several congenic mouse strains. The response studied, the IgM response to the haptenated synthetic polypeptides is clearly T-helper cell dependent (Fig. 4). For both synthetic polypeptides it was possible to induce antigen specific helper cells from unprimed T cells obtained from spleen and lymph node (Figs 1, 2 and 3) and from cortisone resistant thymocytes (data not shown).

To verify whether the induction was just as effective in non-responders and low responders as in high responder mice, the critical parameters for HC induction were titrated. There was no significant difference in antigen concentration required, in the optimum time for helper cell development, or in the efficiency of HR- and LR- or NR-helper cells once induced. These experiments effectively rule out the possibilities that in our mouse strains with our antigens, there was either a qualitative defect in HC induction with unprimed T cells, or that there was a quantitative defect in the helper cells produced. These results are similar to those previously reported using either the limiting dilution approach (Lichtenberg *et al.*, 1974) or antigen specific T-cell factors (Taussig, 1974), which demonstrated that H-2^k mice, low responders to (T,G)-A–L had normal T cells, but had a B-cell defect.

It is not yet known what kind(s) of cellular defect NR mice to GAT^{10} exhibit. We are at present investigating to determine whether there exists T- and B-cell diversity of defects similar to that shown by LR mice to (T,G)-A–L.

However our results are somewhat different from those of Kapp *et al.*, 1975, who could only induce *in vivo* or *in vitro* GAT^{10} specific helper cells, using GAT^{10} as antigen in HR mice (C57BL/6J, H-2^b) but not in NR mice (B10.S, H-2^s, or DBA/1, H-2^q); they could only induce GAT^{10} specific helper cells in NR mice by using GAT^{10} conjugated to methylated bovine serum albumin or by presenting the antigen on macrophages. Our results show that both HR strains, B10 and B10.A, and NR strains, DBA/1 and B10.G, to GAT^{10} can be induced to form GAT^{10}-specific helper cells *in vitro* using soluble GAT^{10} as antigen. It is possible that this difference may be due to the assay—Kapp-measured IgG, here IgM is assayed.

It is not clear whether the assay we used in this report which measures anti-hapten plaques, would detect defects in B-cell reactions connected with H-2 linked unresponsiveness to (T,G)-A–L or GAT^{10}; although Katz, Hamaoka & Benacerrat (1972) reported that B cells from non-responder strains to GLT (a copolymer of glutamic acid, lysine and tyrosine) did not respond to DNP conjugated GLT. In order to properly assess B-cell function *in vitro* we are attempting to measure the antibody responses to the polypeptides themselves using (T,G)-A–L (Taussig, 1974) or GAT^{10}-coated sheep erythrocytes (Kapp, Pierce & Benacerraf 1973).

There are many possible lesions which could be responsible for H-2-linked unresponsiveness at the level of T or B cells or macrophages. Experiments are in progress to investigate these possibilities *in vitro*. Since most of the literature on H-2-linked unresponsiveness *in vitro* describes changes in secondary responses, it will be of interest to check such responses *in vitro*, as there may be differences from the primary responses described above, implying that the basis of unresponsiveness may be abnormal regulatory mechanisms. Because the HR and LR or NR strains used in this work (B10, B10.A, B10.Br, B10.G, DBA/1) had normal T-helper cell precursors which could be activated by *in vitro* culture with (T,G)-A–L or GAT, and these helper cells induced normal numbers of IgM antibody-forming cells, these results preclude the presence of any significant quantitative or qualitative receptor differences between virgin T cells of the HR and LR or NR strains used.

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