Immunology 1987 **61** 475–482

Distribution and functional characteristics of antigen-specific helper T cells arising after Peyer's patch immunization

M. L. DUNKLEY & A. J. HUSBAND *Faculty of Medicine, The University of Newcastle, Newcastle, New South Wales, Australia*

Accepted for publication 26 March 1987

SUMMARY

Antigen-specific T-helper cells for IgA responses arise in Peyer's patches (PP) following their immunization by subserosal injection of keyhole limpet haemocyanin (KLH). These are of the W3/25 phenotype and the W3/25 receptor is shown here to be involved in their helper function. These cells originate in PP and migrate via mesenteric lymph nodes (MLN) to thoracic duct lymph, although the MLN appear to be unnecessary for the induction or maturation of antigen-specific helper cells collected in thoracic duct lymph after intra-Peyer's patch (i.p.p.) immunization. KLH-specific helper cells can be detected subsequently in the intraepithelial lymphocyte population and also among lamina propria lymphocytes. The helper cells also relocate to PP distant to their site of origin where they are retained only when antigen is present. While i.p.p. immunization is an efficient route for the induction of IgA helper cells in gut-associated lymphoid tissue, it differs from oral immunization in that concomitant induction of antigen-specific splenic suppressor cells does not occur, indicating a role for epithelial antigen processing in this phenomenon.

INTRODUCTION

Mucosal sites are characterized by the predominance of IgA antibody production in response to local immunization, but whether this is a function of T-cell directed differentiation of B-cell precursors (Kawanishi, Saltzman & Strober, 1983) or is a reflection of the extent of their antigen-driven proliferation at mucosal sites (Cebra *et al.*, 1983) remains controversial. It is well documented, however, that IgA effector responses are highly T-cell dependent (Clough, Mims & Strober, 1971; Crewther & Warner, 1972; Pritchard, Riddaway & Micklem, 1973), and populations of T cells that provide exclusive help for IgA production have been identified in humans (Endoh *et al.*, 1981; McCaughan, Adams & Basten, 1984) and rodents (Richman *et al.*, 1981; Dunkley & Husband, 1986).

Most studies of T-cell migration have been undertaken either with bulk populations, representing a wide range of specificities, the behaviour of which reflects the sum of a number of potential influences, or with cloned antigen-specific cells which may display aberrant migration patterns (Dailey *et al.*, 1982). In order to overcome these deficiencies we have attempted to study the origins and migration patterns *in vivo* of a functional population of antigen-specific T cells providing isotype-specific help.

We have previously shown that immunization of rat Peyer's patches (PP) with keyhole limpet haemocyanin (KLH) gives rise to antigen-specific helper cells for IgA responses in gut-associated lymphoid tissue (GALT) and in thoracic duct lymph but not peripheral lymph nodes (PLN) (Dunkley & Husband, 1986). These findings suggest that T-helper cells arising after GALT immunization display a mucosally restricted distribution pattern and highlight the need for appropriate antigen localization to ensure successful IgA responses.

While PP have been identified as an enriched source of these cells, it is unknown whether mesenteric lymph nodes (MLN) also contribute to the population of T-helper cells arising after intestinal immunization. It is also unclear whether these cells provide help for IgA responses only within the PP affector sites or whether they also disseminate into intestinal effector sites (lamina propria and epithelium) where they may play a role in the retention and proliferation of antigen-specific IgA B-cell precursors which migrate to immunized segments of intestine (Husband, 1982).

In this paper we extend our previous studies of the origin and migration of *in vivo*-generated antigen-specific T-helper cells for IgA, IgG and IgM responses to KLH, identified by their ability

Abbreviations: BGG, bovine gamma globulin; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DNP, dinitrophenyl; FCS, fetal calf serum; GALT, gut-associated lymphoid tissue; i.d. intraduodenal; IEL, intraepithelial lymphocytes; i.p. intraperitoneal; i.p.p., intra-Peyer's patch; KLH, keyhole limpet haemocyanin; LPL, lamina propria lymphocytes; MLN, mesenteric lymph nodes; OVA, ovalbumin; PFC, plaque-forming cells; PLN, peripheral lymph nodes; PP, Peyer's patch; TDL, thoracic duct lymphocytes; TNP, trinitrophenyl.

Correspondence: Dr A. J. Husband, Faculty of Medicine, The University of Newcastle, Newcastle, NSW 2308, Australia.

to support *in vitro* the response of DNP-primed B cells to TNP-KLH. The phenotype of the T-helper cells generated by immunization of PP are further characterized and the role of antigen in their distribution is investigated.

MATERIALS AND METHODS

Animals

Male rats of the inbred PVG strain were used at 12–16 weeks of age for all experiments.

Antigens

KLH, dinitrophenylated KLH (DNP-KLH) (492 DNP groups per protein molecule based on a molecular weight of 2,000,000) and dinitrophenylated bovine gamma globulin (DNP-BGG) (50 mol DNP per mol of globulin based on a molecular weight of 17,530,000) were obtained from Calbiochem, Sydney, Australia. Ovalbumin (OVA) Grade V was obtained from Sigma, St Louis, MO. Trinitrophenylated OVA (TNP-OVA) was prepared with OVA Grade V (Sigma) and picryl sulphonic acid (TNBS) Grade I (Sigma) as described previously (Dunkley & Husband, 1986). The coupling ratio was 12 molecules of TNP per molecule of OVA.

Immunizations

Intra-Peyer's patch (i.p.p.) immunization was achieved by injection of approximately 5 μl of KLH or OVA (100 μg/ml) in complete Freund's adjuvant (CFA) under the serosa overlying PP in the small intestine. Immunization of all PP in the small intestine constituted a total dose of approximately 10 μg protein antigen per rat. Immunized PP were occasionally larger but otherwise macroscopically and microscopically similar in appearance to unimmunized PP. Rats were either killed 2 weeks after i.p.p. immunization or in some experiments they were given an intraduodenal (i.d.) immunization 1 or 2 weeks after i.p.p. immunization and killed 5 days later.

Surgical preparations

Thoracic lymphatic duct cannulations were performed as described by Gowans & Knight (1964), and lymph was collected overnight at room temperature into sterile flasks containing 5 ml PBS to which was added 20 U/ml heparin, 2000 U/ml penicillin and 2000 μg/ml streptomycin.

Double Thiry-Vella loops of isolated intestinal segments, with mesentery and blood supply intact, were prepared as previously described (Husband, 1982).

Preparation of cell suspensions

Thoracic duct lymphocytes (TDL) were washed twice in phosphate-buffered (pH 7·3) saline (PBS) containing calcium and magnesium and supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml fungizone. Single cell suspensions were prepared from PP and spleen as described previously (Dunkley & Husband, 1986). Lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) were prepared by a modification of the method described by Lyscom & Brueton (1982). The small intestine was rinsed thoroughly with PBS at 4° and then placed in fresh cold PBS. The PP were then excised and placed in cold PBS and the remaining intestine cut into 1-cm lengths. These were slit open longitudinally and the mucus gently scraped off. The pieces were then washed vigorously in citrate buffer, pH 7·2 (containing penicillin, streptomycin and fungizone), and placed in a 50-ml centrifuge tube containing 30 ml of fresh citrate buffer. They were then rotated for 3 min in 1 mM dithiothreitol in citrate buffer, washed twice in citrate buffer and placed in 25 ml of warm citrate buffer containing antibiotics and 2·5% bovine serum albumin (BSA) at 37° and rotated for 15 min. The tubes were then vortexed vigorously for 30 seconds to dislodge the epithelial cells, and the resulting suspension was eluted with PBS through cotton wool to remove mucus and debris. The cells were centrifuged at 400 ***g*** for 7 min and resuspended in supplemented PBS.

In order to obtain LPL the remaining gut was chopped on a sieve using a scalpel blade and gently pushed through the sieve into citrate buffer (containing antibiotics and BSA). The resulting suspension was eluted on cotton wool columns to remove mucus and debris, then centrifuged (400 ***g***, 7 min) and washed in supplemented PBS. On some occasions the chopped gut pieces were first incubated in collagenase (20 U/ml) for 15 min at 37° but this did not improve the cell yield.

Lymphocyte suspensions to be assayed for helper activity (with the exception of LPL) were enriched for T cells using nylon-wool columns (Julius, Simpson & Herzenberg, 1973). LPL cell yields were too low to allow nylon-wool purification.

In some experiments nylon-wool treated T cells were further purified by subset depletion using a modification of the panning technique of Wysocki & Sato (1978). Plastic petri-dishes (6-cm diameter, Lab-Tek, Miles Laboratories, Sydney, Australia) were coated by overnight incubation at 4° with rabbit anti-mouse IgG (Dako Immunoglobulins, Copenhagen, Denmark) diluted 1/100 in borate buffer (pH 8·7). The following day the plates were rinsed once with PBS and then three times with PBS containing 1% FCS. Cells to be panned were pretreated for 30 min at 4° with the monoclonal antibodies W3/25 (recognizing predominantly helper/inducer phenotype) diluted 1/20, or OX8 (recognizing predominantly cytotoxic/suppressor phenotype) diluted 1/50. The monoclonal antibodies were obtained as ascites fluid from SeraLab (CSL, Melbourne, Australia). The cells were washed twice and resuspended to 15×10^6 cells/ml. A 2-ml volume of this suspension was added to each of the plates, which were then incubated for 90 min at 4° with gentle swirling each 30 min, after which the non-adherent cells were gently aspirated with the supernatant. Populations of T-cell subsets stained with subset-specific monoclonal antibodies and $F(ab')_2$ fragments of fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Westchester, PA) were enumerated by flow cytometry using an Ortho Spectrum III flow cytometer.

Assay of helper cell activity

KLH-specific helper activity was assayed as described previously (Dunkley & Husband, 1986). Briefly, an *in vitro* microculture system was used to determine the ability of T cells from KLH-immunized rats to help DNP-primed B cells respond to DNP-KLH. In some experiments OVA-specific helper activity was assayed by determining the ability of T cells to help DNP-primed B cells respond to DNP-OVA. DNP-primed B cells were obtained from spleens of syngeneic rats immunized 3–4 weeks previously by intraperitoneal (i.p.) injection of 100 μg DNP-BGG in CFA. Helper activity of carrier-primed T cells was expressed as the number of anti-DNP plaque-forming cells (DNP-PFC) per 10^6 DNP-primed spleen cells cultured.

RESULTS

Antigen specificity of helper cells in PP after i.p.p. immunization

We have shown previously that T-helper cells detected in MLN following i.p. immunization with KLH or OVA are specific for the immunizing antigen (Dunkley & Husband, 1986). It was necessary to determine whether this also applies to helper cells detected in PP after i.p.p. immunization. PP cell suspensions were prepared from rats 2 weeks after i.p.p. immunization with either KLH or OVA in CFA or with saline in CFA and tested for their ability to provide help for an *in vitro* anti-DNP response among DNP-primed B cells cultured with either DNP-KLH or TNP-OVA (Fig. 1). PP cells from rats immunized with KLH

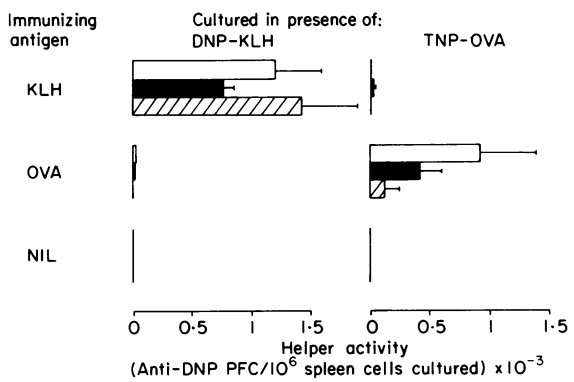

Figure 1. Antigen specificity of the helper T-cell response in PP following i.p.p. immunization. The capacity of PP T cells to provide help for an anti-DNP response among DNP-primed spleen cells cultured with either DNR-KLH or TNP-OVA was assayed 2 weeks after i.p.p. immunization of PP donor rats with either KLH, OVA or PBS emulsified in CFA. The help provided for IgM (□), IgA (■) and IgG (■) anti-DNP responses is shown at a T cell:spleen cell ratio of 1:1. Histograms represent means of triplicate cultures from each of two rats in each group, and vertical bars are standard errors.

provided help for IgA, IgM and IgG responses in the presence of DNP-KLH, but did not provide help for responses with TNP-OVA. Conversely, PP cells from animals immunized with OVA provided help when cultured with TNP-OVA but not with DNP-KLH. PP cells from animals immunized with CFA alone did not provide help for an anti-DNP response with either DNP-KLH or TNP-OVA. Thus, the helper cells produced in PP after i.p.p. immunization are specific for the immunizing antigen and do not provide help for antigens with which the rats have not been previously immunized.

Phenotype of KLH-specific helper cells in PP of i.p.p. immunized rats

While antigen-specific functional help has been demonstrated among PP T cells, the phenotype of these cells required verification to provide a basis for further experiments involving subset depletion using subset-specific monoclonal antibodies. Since the monoclonal antibody W3/25 has been shown to identify helper T cells for anti-hapten responses in rats (White *et al.*, 1978), this antibody was used to determine whether the helper cells in PP of i.p.p. immunized rats display the W3/25 phenotype. Initially, attempts to purify W3/25$^+$ cells from PP suspensions by panning were unsuccessful due to non-specific binding of PP cells to the dishes. Treatment of cell populations with W3/25 and complement was also unsuccessful, perhaps because the antigen that the W3/25 antibody recognizes is sparsely distributed on the cell membrane (Webb, Mason & Williams, 1979) or because this monoclonal antibody does not fix complement effectively. The addition of W3/25 antibody to cultures has, however, been shown to inhibit *in vitro* mixed lymphocyte responses in rats (Webb *et al.*, 1979). This strategy was therefore used to study the effects of W3/25 antibody on the *in vitro* helper activity of PP cells from rats immunized i.p.p. with KLH.

Mouse ascites containing monoclonal antibodies W3/25 or OX8, eluted on columns of Sephadex G-25 (Pharmacia, Uppsala, Sweden) to remove azide preservative, were sterile-filtered and then added to PP helper assay cultures at a final dilution of 1/100 of ascites fluid. This dilution was calculated to provide an antibody concentration equivalent to that used by Webb *et al.* (1979) to inhibit mixed lymphocyte responses. The data in Fig. 2 indicate that the addition of W3/25 antibody abrogated the helper capacity of PP cells for anti-DNP responses of all isotypes, whereas, the addition of OX8 antibody had no effect.

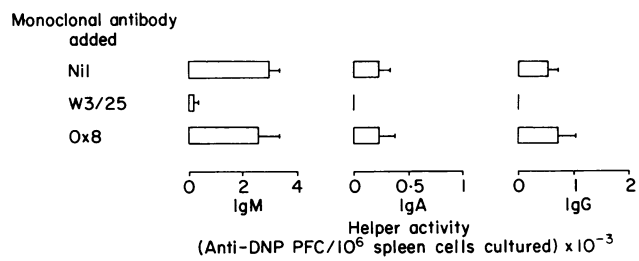

Figure 2. Phenotype of KLH-specific helper cells in PP following i.p.p. immunization with KLH. T-cell suspensions were prepared from PP 2 weeks after immunization, and their capacity to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH. The effect of the addition of W3/25 or OX8 monoclonal antibodies at a final dilution of 1/100 of ascites fluid was determined. Histograms represent means of triplicate cultures from each of four rats in each experiment, and horizontal bars are standard errors.

This not only confirms that KLH-specific helper cells appearing in PP after i.p.p. immunization are W3/25$^+$ T cells, but also implies that the W3/25 receptor is involved in the helper function of these cells. The lack of effect of OX8 antibody indicates that the results obtained for W3/25 do not represent non-specific effects of the addition of mouse antibody *per se* and also, as the helper activity did not increase after the addition of OX8, either that antigen-specific suppressor cells are not present in PP after i.p.p. immunization, or that the OX8 receptor is not involved in suppressor function.

Origin of helper cells in thoracic duct lymph

Following i.p.p. immunization with KLH, antigen-specific helper activity appears in PP, reaching a peak at about 14 days (Dunkley & Husband, 1986). A similar pattern but of lower

magnitude occurs in MLN and thoracic duct lymph, whereas no activity was demonstrated in PLN.

The presence of helper activity among TDL demonstrates that helper cells generated by i.p.p. immunization are a migrating population that probably originates in PP and/or MLN. In order to determine the extent to which MLN contribute to the pool of helper T cells among TDL after i.p.p. immunization, the helper activity was examined in the thoracic duct lymph of rats from which MLN had been removed several months prior to i.p.p. immunization. The helper activity for all isotypes was not altered significantly by mesenteric lymphadenectomy (Fig. 3), suggesting that MLN are not necessary for the induction or maturation of KLH-specific helper T cells appearing among TDL after i.p.p. immunization and do not contribute substantially to the migrating pool of these cells.

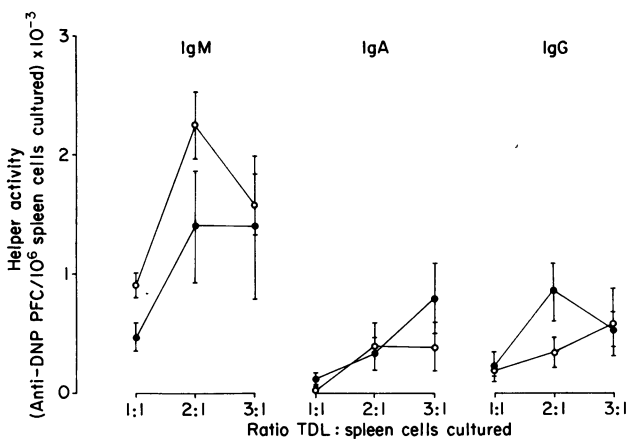

Figure 3. Effect of mesenteric lymphadenectomy on specific helper activity of TDL. The thoracic duct was cannulated in normal (●) or mesenteric lymphadenectomized (○) rats 2 weeks after i.p.p. immunization with KLH. T cells were prepared from an overnight collection of TDL, and their capacity to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH. Plotted points represent means of triplicate cultures from each of three normal and three lymphadenectomized rats at the T cell:spleen cell ratios shown. Vertical bars represent standard errors.

Migration of helper cells to effector sites

It is well documented that IgA plasma cell precursors responding to GALT immunization arise in PP and migrate via the thoracic duct and blood circulation to subepithelial effector sites (Husband, 1982). The destination of the PP-derived T-helper cells appearing in thoracic duct lymph and entering the circulation is important in terms of their sites of ultimate effector function. In view of the demonstrated occurrence of cells of the helper phenotype in the gut lamina propria and epithelium (Lyscom & Brueton, 1982), KLH-specific helper cell activity was determined at these sites in rats that had been immunized i.p.p. 2 weeks previously with KLH. Preparations of IEL usually yielded approximately 15–20 × 10^6 lymphocytes per rat, however, the preparation of LPL was more difficult and usually yielded only 1–5 × 10^6 lymphocytes.

Histological examination of sections of gut after IEL preparation demonstrated that the lamina propria remained substantially intact, although contamination of IEL with some LPL could not be excluded, particularly since some epithelium usually remained at the base of villi. The IEL preparations contained on average 37% viable lymphocytes and 20% viable epithelial cells (determined by trypan blue exclusion), and LPL preparations contained 41% viable lymphocytes and 11% viable epithelial cells.

The helper activity in these preparations is shown in Fig. 4.

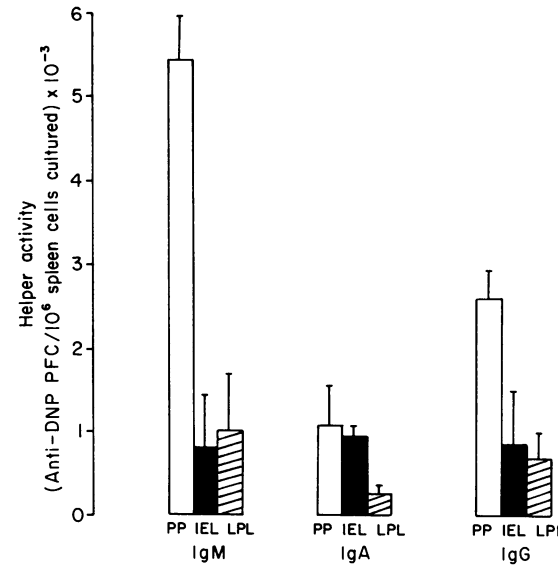

Figure 4. Specific helper activity in gut IEL and LPL after i.p.p. immunization. T-cell suspensions were prepared from PP (□), IEL (■) and LPL (▨) 2 weeks after i.p.p. immunization with KLH, and their capacity to provide help for anti-DNP responses of IgM, IgA and IgG isotypes was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH. Histograms represent means of triplicate cultures from each of 14 rats for PP, four rats for IEL and three rats for LPL at T cell:spleen cell ratios of 2:1 for PP and IEL, and 1:1 for LPL. Vertical bars are standard errors.

Helper activity was present in both IEL and LPL fractions although the levels were low and on some occasions were undetectable. Whether this was due to cell damage during preparation or is a true reflection of biological variation is not clear. Nevertheless, these data suggest that KLH-specific helper cells do occur at both of these effector sites after i.p.p. immunization since in unimmunized rats no helper activity is detectable (data not shown).

Role of lumenal antigen in helper cell distribution in PP

The effect of whole gut lumenal challenge in rats given segmental i.p.p. immunization was investigated to determine whether helper cells migrate between PP and the role of antigen in their distribution. PP in the proximal half only or in the distal half only were immunized by subserosal antigen injection and the helper activity examined in cells pooled from either proximal or distal PP. In some animals in which distal PP were immunized, a second dose of antigen in saline was administered to the whole length of small intestine by i.d. injection (accessed by laparotomy) of antigen in saline.

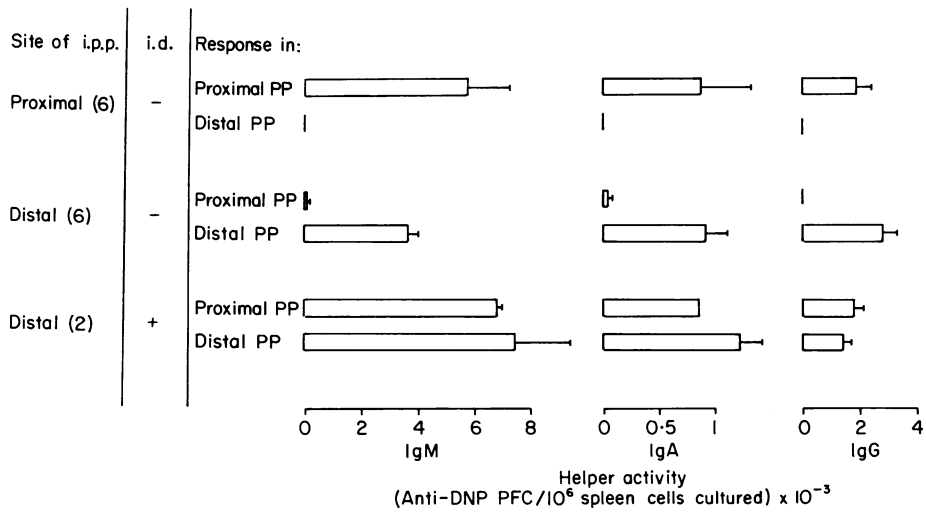

Figure 5. Specific helper activity in PP after segmental i.p.p. immunization. T-cell suspensions were prepared from proximal or distal PP 2 weeks after immunization with KLH of only proximal or only distal PP. In some rats a secondary lumenal dose of KLH was given by i.d. injection 1 week after IPP. The capacity of these cells to provide help for anti-DNP responses of such isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH at a T cell: spleen cell ratio of 2: 1. Histograms represent means of triplicate cultures from each of the number of rats shown in parentheses. The horizontal bars are standard errors.

The results (Fig. 5) show that, in the absence of secondary lumenal stimulation substantial helper activity was detected only in immunized PP. However, after a secondary dose of KLH given i.d., substantial helper activity was detected in both immunized and non-immunized PP. The presence of helper T cells in non-immunized PP could not be attributed to the i.d. dose of antigen alone as we have shown previously that i.d. immunization does not stimulate detectable helper activity in PP (Dunkley & Husband, 1986). These data suggest that T-helper cells arising in PP migrate between PP but only accumulate in detectable numbers when antigen is present.

The above data also indicate that i.d. immunization does not augment the helper activity of previously immunized PP. In order to obtain further evidence for this, the effect of segmental lumenal challenge in rats with all PP immunized was investigated using rats bearing double Thiry-Vella loops constructed 10 days after total i.p.p. immunization. Four days later the distal loop only in each rat was infused intralumenally with KLH in saline and the helper activity in PP in both proximal and distal loops assayed (Fig. 6). The helper activity was similar in PP of both loops, suggesting that i.p.p. immunization by subserosal injection of antigen in CFA yields a maximal T-helper cell response in PP that cannot be expanded further by subsequent lumenal antigen.

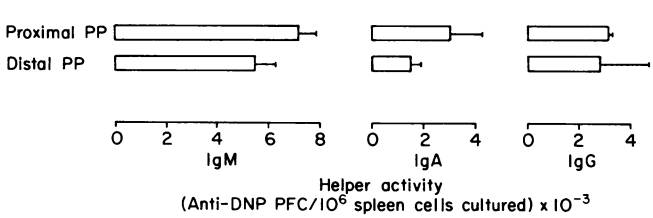

Figure 6. Specific helper activity in immunized PP after segmental lumenal challenge. Thiry-Vella loops were prepared in rats 1 week after i.p.p. immunization of all PP with KLH, and 4 days later the distal loop only was infused with KLH. After a further 5 days the capacity of T cells prepared from PP in proximal or distal loops to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH at a T cell: spleen cell ratio of 2: 1. Histograms represent means of triplicate cultures from each of three rats, and horizontal bars are standard errors.

Absence of KLH-specific suppressor cells in spleen after i.p.p. immunization

The kinetics of the helper activity in spleen after i.p.p. immunization was compared with that in PP (Fig. 7) and, although slight helper activity was present 7 days after immunization, there was no activity by Day 14. The possibility that the low net helper activity observed reflected the presence of suppressor cells rather than the absence of helper cells was investigated in three ways.

In the first experiment spleen cells from either normal or i.p.p. immunized rats were added to helper assay cultures of PP cells from i.p.p. immunized rats (Fig. 8). A reduction in helper activity was observed whether the added spleen cells were from an i.p.p. immunized rat or from a normal rat. This reduction in helper activity did not indicate antigen-specific suppression but probably represented a crowding effect.

Secondly, OX8 antibody was added to helper assay cultures of spleen cells (at a final dilution of 1/100 of ascites fluid). This had no effect on the level of help detected among spleen cells (Table 1), indicating that no helper cells were present, that no KLH-specific suppressor cells were present, or that the OX8 receptor is not involved in suppressor functions.

Thirdly, spleen cells from i.p.p. immunized rats were depleted of $OX8^+$ cells by panning in an attempt to determine whether their presence may have been interfering with the activity of helper cells in the spleen (Table 2). Panning reduced the proportion of $OX8^+$ cells from 21·0 to 0·5%. Although the panned cells showed a two-fold increase in helper activity for an IgM response when compared with the starting population, there was no difference for IgA or IgG responses. The enhanced IgM response probably reflected the corresponding enrichment of $W3/25^+$ cells occurring as a result of panning.

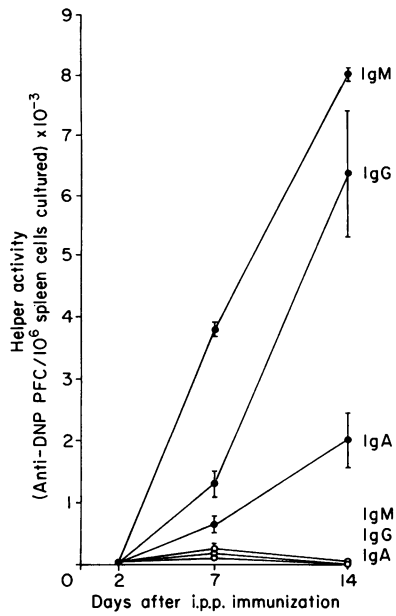

Figure 7. The kinetics of specific helper activity in spleen after i.p.p. immunization. The capacity of T-cell suspensions prepared from PP (●) and spleen (○) at various times after i.p.p. immunization with KLH to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH. Cells were cultured at a T cell:spleen cell ratio of 2:1. Plotted points represent means of triplicate cultures from each of three rats at each time-point, and vertical bars are standard errors.

Taken together, the above observations indicate that neither helper nor suppressor cells specific for KLH appear in spleen in significant numbers following i.p.p. immunization with KLH.

DISCUSSION

The experiments reported here have demonstrated that antigen-specific helper cells of W3/25 (helper/inducer) phenotype arise in PP of rats after i.p.p. immunization with KLH and subsequently appear in thoracic duct lymph, gut epithelium and gut lamina propria.

Inferences regarding helper/inducer phenotype have been drawn from the demonstrated abrogation of helper function in the presence of W3/25 monoclonal antibody (Fig. 2). This technique has been widely used to detect the involvement of cell surface molecules in cell function (van Seventer *et al.*, 1986; Fleischer, Schrezenmeier & Wagner, 1986). Its validity is reinforced by the work of Webb *et al.* (1979) who showed that antibody derived from the same clone as that used here inhibited mixed lymphocyte responses in rats, and that this was a specific effect and not the result of antibody-induced agglutination or cell death. Indeed, mitogen-induced T-cell proliferation was unaffected by the addition of W3/25 antibody at 100 times the level required to abrogate helper function.

The failure of mesenteric lymphadenectomy to affect significantly the helper activity among TDL indicates that helper cells arising from this immunization regime are generated solely in the intestine (presumably the PP) and that MLN are unnecessary for their induction or maturation.

The appearance of antigen-specific helper T cells among TDL and the subsequent detection of activity among IEL and LPL populations suggest that helper cells may migrate to effector sites via the thoracic duct and blood circulation, the pathway described for B-effector cells arising after i.p.p. immunization (Husband & Dunkley, 1985). However, this observation is not in itself sufficient evidence to indicate that these cells do migrate to effector sites via the thoracic duct. On the contrary, Jeurissen, Sminia & Kraal (1984) suggest that PP-derived T-helper cells are able to migrate directly from PP to the surrounding villus lamina propria without entering lymph. Confirmation of a pathway involving the thoracic duct is being sought in current experiments in this laboratory observing the effects of chronic thoracic duct drainage on IEL- and LPL-specific helper activity after i.p.p. immunization.

The higher helper activity in IEL compared with LPL is surprising in view of reports that similar numbers of W3/25$^+$ cells occur at both these sites and that OX8$^+$ (suppressor/cytotoxic subset) cells predominate over W3/25$^+$ cells in rat IEL (Lyscom & Brueton, 1982). However, due to low cell recoveries, LPL preparations may not have provided a representative population, and the lower levels demonstrated in this location may reflect the difficulty in obtaining cells from this site. Cross-contamination of IEL and LPL also cannot be excluded.

There was large variation between animals in the helper activity in both LPL and IEL fractions, probably because of variations in the extent of cell damage and the amount of mucus present, which tended to clump the cells. Another potential source of variation could have arisen from a failure to remove all PP from the gut prior to the preparation of LPL and IEL suspensions. However, this is not likely to have contributed significantly to the activity detected since any PP small enough to have escaped excision during preparation of the gut for LPL and IEL would probably not have been immunized initially. In this regard, we have demonstrated in this paper that helper cells are not detectable in non-immunized PP unless a secondary i.d. immunization is given. Nevertheless, more refined isolation methods for the study of IEL and LPL cell activities are required. Future studies should also investigate the time-course of appearance of helper cell function at effector sites as the single time-point chosen in these studies (2 weeks post-immunization) may not have been optimal for these locations.

The presence of antigen-specific T-helper cells in both affector (PP) and effector sites (gut lamina propria and epithelium) after GALT immunization is in contrast to the distribution of B cells responding to a similar immunization regime (Husband & Dunkley, 1985). While IgA B-cell precursors arise in PP, they do not express effector function (antibody production) until they leave this site. Functional T-helper cells are clearly required in PP for the induction of B-cell responses to antigen presented to PP, but the expression of helper function by T cells in effector sites is probably not involved in induction but may be one of the factors that determine the retention and expansion of antigen-specific IgA blast cells described in immunized gut lamina propria (Husband, 1982).

The role of antigen in the induction and relocation of T-helper cells was investigated by segmental immunization experiments. In experiments where only some PP of the rat intestine were immunized, the lack of helper activity in unimmunized PP revealed the importance of antigen localization in PP for the generation of a T-helper cell population. This finding was also thought to imply that T-helper cells do not relocate to distant PP. However, a secondary lumenal challenge with KLH via the

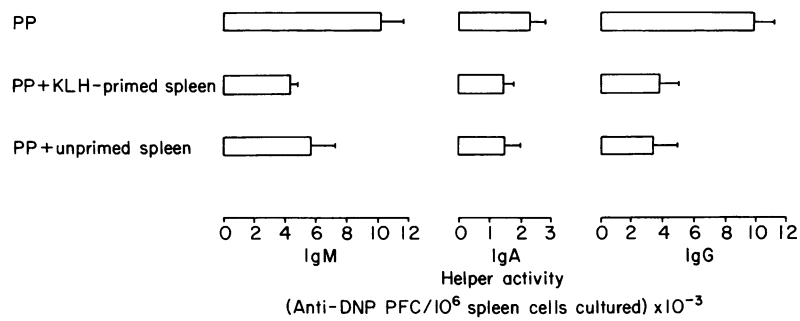

Figure 8. Effect of spleen cells on helper activity in Peyer's patches of i.p.p. immunized rats. T cells were prepared from PP of rats immunized i.p.p. 14 days previously with KLH and were cultured either alone, with autologous splenic T cells, or with splenic T cells from unimmunized rats. The capacity of these combinations of cells to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH. Cells were cultured at a PP T cell: splenic T cell: DNP-primed spleen cell ratio of 1:1:1. Values are means (± standard error) of triplicate cultures of cells from three rats.

Table 1. Effect of addition of OX8 antibody on helper activity among splenic T cells of i.p.p. immunized rats

Source of T cells	Monoclonal antibody added	T cell: spleen cell ratio	Helper activity (anti-DNP PFC/10^6 spleen cells cultured) IgM	IgA	IgG
Spleen	Nil	1:1	15±14	80±85	66±39
		2:1	10±10	0	0
Spleen	OX8	1:1	0	0	0
		2:1	0	0	0
PP	Nil	1:1	996±163	129±92	823±370
		2:1	5640±832	329±38	1916±630

T cells were prepared from the spleens of rats immunized i.p.p. with KLH 14 days previously and the effect of OX8 antibody on their capacity to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH. Helper activity among autologous PP cells is shown for comparison. Cells were cultured at the T cell: DNP-primed spleen cell ratios shown. Values are means (± standard error) of triplicate cultures from each of three rats.

Table 2. Effect of depletion of $OX8^+$ cells on phenotype and helper activity among splenic T cells from i.p.p. immunized rats

	% cells staining with: W3/13	W3/25	OX8	Anti-IgG
Before panning	70·4	54·2	21·7	11·2
After panning	78·6	79·9	0·5	5·2
Ratio panned: unpanned	1·2	1·5	0·2	0·5

	Helper activity (anti-DNP PFC/10^6 spleen cells cultured) IgM	IgA	IgG
Before panning	144±8	5±5	202±21
After panning	320±73	2±2	192+123

T cells were prepared from the spleens of rats immunized i.p.p. with KLH 14 days previously. The surface marker phenotypes were determined by flow cytometry after staining with the appropriate antibody and FITC-conjugated anti-IgG. The effect of depletion of $OX8^+$ cells on the capacity to provide help for anti-DNP responses of each isotype was assayed by culturing with DNP-primed spleen cells in the presence of DNP-KLH at a T cell: spleen cell ratio of 2:1. Values are means (± standard error) of triplicate cultures from each of three rats.

i.d. route (which alone stimulates no helper activity in PP) gave rise to substantial helper activity in the previously unimmunized PP, suggesting that helper cells do migrate between PP but only accumulate in detectable numbers when antigen is present.

The data presented in this paper also extend our previous studies of the systemic migration of mucosally generated T-helper cells. We previously showed an apparent mucosal restriction of helper cells following i.p.p. immunization, in that little or no response was detectable in PLN. We have now demonstrated that the helper activity in spleen is also low after i.p.p. immunization, and this provides further evidence for the mucosally restricted relocation of T-helper cells responding to i.p.p. immunization.

The lack of helper activity in spleen was not due to the presence of suppressor cells, and in this respect i.p.p. immunization appears to differ from oral immunization. There have been numerous reports of antigen-specific suppressor cells appearing in spleen after oral immunization with soluble antigens (Mattingly & Waksman, 1978, 1980; MacDonald, 1982). These appear under the influence of suppressor inducer cells from PP which migrate to the spleen and generate suppressor effector cells at that site (Mattingly, 1984; Gautam & Battisto, 1985). The failure to detect antigen-specific suppressor activity in either PP (Fig. 2) or spleen suggests that i.p.p. immunization with KLH in CFA does not activate this suppressor circuit. Since the i.p.p. immunization route provides antigen access to PP without involvement of the gut epithelium, the role of epithelial antigen processing in induction of suppression to orally administered antigens should be investigated. In this respect it is of interest that epithelial cells have been reported to be involved in antigen presentation to T cells (Cerf-Bensussan *et al.*, 1984; Barclay & Mason, 1982; Bland & Warren, 1986a) and as such are potent suppressor cell inducers (Bland & Warren, 1986b).

It is also possible that the use of CFA for antigen delivery to PP affected the induction of suppression since it is a potent inducer of cell-mediated immunity. An alternative biodegradable adjuvant has been developed that stimulates equivalent IgA responses after intestinal immunization without persistent lesions (Husband & Dunkley, 1987), and it will be of interest to compare the balance of helper and suppressor responses obtained with this adjuvant.

This paper provides further evidence for the hypothesis that IgA responses occurring after immunization of the intestine are a reflection as much of the activities and locations of regulatory T cells as of the B-cell precursors of antibody-containing cells. The T cells providing help for these responses originate in PP and relocate to both affector and effector sites in the intestine, and to some extent their distribution at these sites appears to be antigen dependent.

ACKNOWLEDGMENT

This work was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

BARCLAY A.N. & MASON D.W. (1982) Induction of Ia antigen in rat epidermal cells and gut epithelium by immunological stimuli. *J. exp. Med.* **156,** 1665.

BLAND P.W. & WARREN L.G. (1986a) Antigen presentation by epithelial cells of the rat small intestine. I. Kinetics, antigen specificity and blocking by anti-Ia antisera. *Immunology*, **58,** 1.

BLAND P.W. & WARREN L.G. (1986b) Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. *Immunology*, **58,** 9.

CEBRA J.J., CEBRA E.R., CLOUGH E.R., FUHRMAN J.A. & SCHWEITZER P.A. (1983) Relationships and regulation of IgG, IgE or IgA expression during clonal outgrowth of primed B cells. In: *Regulation of the Immune Response*, 8th Int. Convoc. Immunol. Amherst, NY, 1982 (eds P. L. Ogra and D. M. Jacobs), p. 107. Karger, Basel.

CERF-BENSUSSAN N., QUARONI A., KURNICK J.T. & BHAN A.K. (1984) Intraepithelial lymphocytes modulate Ia expression by intestinal epithelial cells. *J. Immunol.* **132,** 2244.

CLOUGH J.D., MIMS L.H. & STROBER W. (1971) Deficient IgA antibody responses to arsanilic acid bovine serum albumin (BSA) in neonatally thymectomized rabbits. *J. Immunol.* **106,** 1624.

CREWTHER P. & WARNER N.L. (1972) Serum immunoglobulins and antibodies in congenitally athymic mice. *Aust. J. exp. Biol. med. Sci.* **50,** 625.

DAILEY M.O., FATHMAN C.G., BUTCHER E.C., PILLEMER E. & WEISSMAN I. (1982) Abnormal migration of T lymphocyte clones. *J. Immunol.* **28,** 2134.

DUNKLEY M.L. & HUSBAND A.J. (1986) The induction and migration of antigen-specific helper cells for IgA responses in the intestine. *Immunology*, **57,** 379.

ENDOH M., SAKAI H., NOMOTO Y., TOMINO Y. & KANESHIGE H. (1981) IgA-specific helper activity of T cells in human peripheral blood. *J. Immunol.* **127,** 2612.

FLEISCHER B., SCHREZENMEIER H. & WAGNER H. (1986) Function of the CD4 and CD8 molecules on human cytotoxic T lymphocytes: regulation of T cell triggering. *J. Immunol.* **136,** 1625.

GAUTAM S.C. & BATTISTO J.R. (1985) Orally induced tolerance generates an efferently acting suppressor T cell and an acceptor T cell that together down-regulate contact sensitivity. *J. Immunol.* **135,** 2975.

GOWANS J.L. & KNIGHT E.J. (1964) The rout of re-circulation of lymphocytes in the rat. *Proc. Roy. Soc. B.* **159,** 257.

HUSBAND A.J. (1982) Kinetics of extravasation and redistribution of IgA-specific antibody-containing cells in the intestine. *J. Immunol.* **128,** 1355.

HUSBAND A.J. & DUNKLEY M.L. (1985) Lack of site of origin effects on distribution of IgA antibody-containing cells. *Immunology*, **54,** 215.

HUSBAND A.J. & DUNKLEY M.L. (1987) A vaccination model for stimulating intestinal IgA responses. *Vet. Immunol. Immunopath.* (in press).

JEURISSEN S.H.M., SMINIA T. & KRAAL G. (1984) Selective emigration of suppressor T cells from Peyer's patches. *Cell Immunol.* **85,** 264.

JULIUS M.H., SIMPSON E. & HERZENBERG L.A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3,** 645.

KAWANISHI H., SALTMAN L.E. & STROBER W. (1983) Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. I. T cells derived from Peyer's patches that switch sIgM B cells to sIgA B cells *in vitro*. *J. exp. Med.* **157,** 433.

LYSCOM N. & BRUETON M.J. (1982) Intraepithelial, lamina propria and Peyer's patch lymphocytes of the rat small intestine: isolation and characterization in terms of immunoglobulin markers and receptors for monoclonal antibodies. *Immunology*, **45,** 775.

MCCAUGHAN G.W., ADAMS E. & BASTEN A. (1984) Human antigen-specific IgA responses in blood and secondary lymphoid tissue: an analysis of help and suppression. *J. Immunol.* **132,** 1190.

MACDONALD T.T. (1982) Immunosuppression caused by feeding. I. Evidence for the activation of a feedback suppressor pathway in the spleens of antigen-fed mice. *Eur. J. Immunol.* **12,** 767.

MATTINGLY J.A. (1984) Immunologic suppression after oral administration of antigen. III. Activation of suppressor-inducer cells in the Peyer's patches. *Cell. Immunol.* **86,** 46.

MATTINGLY J.A. & WAKSMAN B.H. (1978) Immunologic suppression after oral administration of antigen. I. Specific suppressor cells found in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. *J. Immunol.* **121,** 1878.

MATTINGLY J.A. & WAKSMAN B.H. (1980) Immunologic suppression after oral administration of antigen. II. Antigen-specific helper and suppressor factors produced by spleen cells of rats fed sheep erythrocytes. *J. Immunol.* **125,** 1044.

PRITCHARD H., RIDDAWAY J. & MICKLEM H.S. (1973) Immune responses in congenitally thymus-less mice. *Clin. exp. Immunol.* **13,** 125.

RICHMAN L.K., GRAEFF A.S., YARCHOAN R. & STROBER W. (1981) Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. *J. Immunol.* **126,** 2079.

VAN SEVENTER G.A., VAN LIER R.A.W., SPITS H., IVANYI P. & MELIEF C.J.M. (1986) Evidence for a regulatory role of the T8 (CD8) antigen in antigen-specific and anti-T3-(CD3)-induced lytic activity of allospecific cytotoxic T lymphocyte clones. *Eur. J. Immunol.* **16,** 1363.

WEBB M., MASON D.W. & WILLIAMS A.F. (1979) Inhibition of mixed lymphocyte response by monoclonal antibody specific for a rat T lymphocyte subset. *Nature (Lond.)*, **282,** 841.

WHITE R.A.H., MASON D.W., WILLIAMS A.F., GALFRE L. & MILSTEIN C. (1978) T-lymphocyte heterogeneity in the rat: separation of functional subpopulations using a monoclonal antibody. *J. exp. Med.* **148,** 664.

WYSOCKI L.J. & SATO V.L. (1978) Panning for lymphocytes: a method for cell selection. *Proc. natl. Acad. Sci. U.S.A.* **75,** 2844.