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The induction and migration of antigen-specific helper cells for IgA responses in the intestine

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SUMMARY

The distribution of keyhole limpet haemocyanin (KLH)-specific helper cells for antibody responses of IgA, IgM and IgG isotypes in Peyer's patch (PP), mesenteric lymph node (MLN) and peripheral lymph node (PLN) was examined following oral, intraduodenal (ID), intraperitoneal (IP), intra-Peyer's patch (IPP) or subcutaneous (SC) immunization with KLH. Oral or ID immunization gave little or no response in any tissue studied. IP immunization with or without a subsequent ID challenge gave rise to a modest IgA and IgM helper response in MLN but a small IgA and IgM helper response in PP and PLN. IP immunization alone did not stimulate IgG-specific help in any tissues studied, but a small IgG helper response occurred in MLN and PLN after subsequent ID challenge. IPP was the most effective route of immunization, giving rise to a large helper response for IgA, IgM and IgG isotypes in PP, a smaller response in MLN and no response in PLN. The helper response following IPP immunization was not augmented by subsequent ID challenge. SC immunization gave a small but significant helper response for all isotypes in PLN but no response in PP or MLN. The kinetics of the helper response were examined in PP, MLN, PLN and thoracic duct lymph (TDL) following IPP immunization. The helper response for all isotypes in PP was maximal at 2 weeks and then declined. Similar kinetics but of lower magnitude were observed in MLN and TDL. The presence of IgA-specific helper cells in TDL demonstrates that these cells migrate, presumably from GALT, and may constitute an important component of mucosal responses at extraintestinal sites.

INTRODUCTION

IgA antibody-containing cells responding to intestinally presented antigens originate as precursors in Peyer's patches (PP) and migrate through the lymphatic ducts draining the gut-associated lymphoid tissue (GALT) to the mesenteric lymph nodes (MLN) entering the blood circulation via intestinal and thoracic duct lymph (TDL). These cells then leave the circulation in the gut lamina propria and other mucosal sites where they reside as IgA-producing plasma cells (Husband & Gowans, 1978; Husband, 1982).

Less is known about the generation and migration of T cells from GALT. There is evidence to suggest that T cells responding to alloantigens in rats arise in intestinal mucosal tissues as a result of oral stimulation with allogeneic cells (Husband *et al.*, 1984), and kinetic studies suggest that they originate in PP and then appear sequentially in MLN, TDL, gut lamina propria and then distant mucosal sites. They were not detected in systemic lymphoid tissues. Thus, the distribution and migration patterns of this population of T cells appear to be similar to that described for IgA-containing B cells.

However, IgA responses are characteristically dependent on T-cell co-operation (Clough, Mims & Strober, 1971; Crewther & Warner, 1972; Pritchard, Riddaway & Micklem, 1973) which involves other functional subsets of T cells. Populations of T cells that provide exclusive help for IgA responses (T_{Ha}) have been identified in man (Endoh *et al.*, 1981; McCaughan, Adams & Basten, 1984) and mouse (Richman *et al.*, 1981). The cells that regulate IgA responses have been most closely studied in mice, and T cells with both helper (Kawanishi, Saltzman & Strober, 1982, 1983; Kiyono *et al.*, 1982, 1984a, b) and suppressor (Hoover & Lynch, 1983) function have been cloned from PP. Little is known, however, about the distribution of T_{Ha} in GALT, and their origin, site of interaction with antigen and subsequent migration patterns are not clearly identified. Elson, Heck & Strober (1979) have shown that Con A-activated T cells from PP provide a higher level of help for IgA synthesis than spleen or PLN in LPS-driven B-cell cultures, indicating an enrichment of cells with T_{Ha} function in GALT. On the other hand, Arny *et al.* (1984) were unable to substantiate these

Abbreviations: BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; DNP-, dinitrophenylated; FCS, fetal calf serum; GALT, gut-associated lymphoid tissue; ID, intraduodenal; IP, intraperitoneal; IPP, intra-Peyer's patch; KLH, keyhole limpet haemocyanin; MLN, mesenteric lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; PLN, peripheral lymph node; PP, Peyer's patch; SC, subcutaneous; TDL, thoracic duct lymph; T_{Ha}, helper T cells for IgA responses.

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findings with respect to carrier-specific T-cell help generated *in vivo* in PP, MLN and PLN of mice immunized with KLH or SRBC. In these experiments, GALT-derived cells provided no more IgA-specific help than PLN cells after immunization of mice with SRBC, and less after immunization with KLH. Differences in IgA help were revealed only after *in vitro* Con A stimulation of T cells from different sources, after which supernatants from GALT gave greater help than those from spleen.

Since the rat is a more suitable animal than the mouse in which to undertake studies of cell migration, the present study was undertaken, first, to determine whether antigen-specific cells displaying T_{Ha} function can be generated in rats, and second, to obtain information on their location and migration patterns. It has been shown previously that intraperitoneal (IP) or intra-Peyer's patch (IPP) immunization with the soluble antigen ovalbumin (OVA) followed by an intraduodenal (ID) challenge leads to a large IgA-producing B-cell response in the gut lamina propria (Pierce & Gowans, 1975; Husband, 1982; Husband & Dunkley, 1985), and it therefore seemed likely that these immunization routes would provide a useful technique by which to generate an antigen-specific T_{Ha} cell response in GALT. In this paper, we have therefore examined the distribution of keyhole limpet haemocyanin (KLH)-specific helper T cells in GALT (PP and MLN), in TDL and in peripheral lymph nodes (PLN) following immunization with KLH administered orally, ID, IPP or IP (routes expected to give a response in GALT) and also subcutaneously (SC) (a route expected to immunize peripheral lymphoid tissue but not GALT).

MATERIALS AND METHODS

Animals

Male and female rats of the PVG strain were used at 8–16 weeks of age for all experiments.

Antigens

KLH, dinitrophenylated-bovine gamma globulin (DNP-BGG) (50 mol DNP per mol of globulin based on a molecular weight of 17,530,000), and dinitrophenylated-KLH (DNP-KLH) (492 DNP groups per protein molecule based on a molecular weight of 2,000,000) 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 1 (Sigma) according to the method described by Henry (1980) for the preparation of DNP-BGG. The coupling ratio was 12 molecules of TNP per molecule of OVA.

Immunization to produce KLH-specific T-helper cells

ID. 1 week prior to tissue removal, animals were given an ID injection via a small laparotomy of 1 mg of KLH in 1 ml of phosphate-buffered (pH 7·3) saline (PBS) injected directly into the lumen of the duodenum using a 27 gauge needle.

Oral. animals were given KLH (1 mg/ml) in their drinking water for 1 week prior to tissue removal.

IP. Two weeks prior to tissue removal, animals were given an IP injection of 100 μg KLH in 1 ml of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA).

IP+ID. Three weeks prior to tissue removal, animals were given an IP injection of 100 μg KLH in CFA (or 500 μg OVA in CFA) followed 2 weeks later by an ID injection of KLH in PBS (1 mg/ml) or OVA in PBS (5 mg/ml).

IPP. Two weeks prior to tissue removal, KLH (100 μg) in CFA was prepared as for IP injections and a small amount injected under the serosa of each PP as previously described (Husband & Dunkley, 1985).

IPP+ID. Three weeks prior to tissue removal, animals were given IPP immunization with KLH as described above, followed 2 weeks later by an ID injection of KLH in PBS (1 mg/ml).

SC. Two weeks prior to tissue removal, animals were given SC a total of 100 μg KLH in CFA distributed between the footpads, the base of the tail and the scruff of the neck.

Immunization to produce DNP-primed B cells

Three weeks prior to tissue removal, rats were primed by IP injection of 100 μg DNP-BGG in CFA.

Preparation of cell suspensions

MLN, PLN and PP from KLH-primed animals and spleens from DNP-BGG-primed animals were removed aseptically under ether anaesthesia. They were each chopped into small pieces using a scalpel then passed gently through a fine wire mesh sieve into sterile PBS containing 5% heat-inactivated fetal calf serum (FCS) and penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 0·25 μg/ml (obtained as a 100 × concentrate mixture from M. A. Bioproducts, Walkersville, MD). The PP cell suspension was immediately passed through a cotton wool filter to remove clumps, mucus and debris. Clumps were removed from MLN and PLN by standing for 10 min. The cell suspensions were then centrifuged, the cells resuspended in PBS and adjusted to 50×10^6 cells/ml.

Cells were collected from TDL of KLH-primed animals

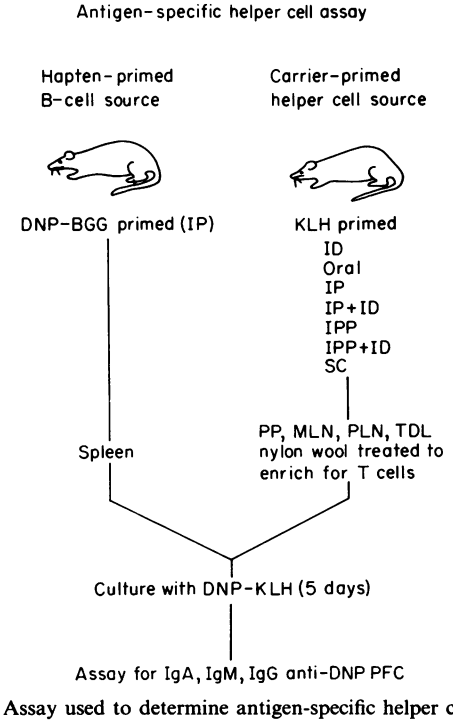

Figure 1. Assay used to determine antigen-specific helper cell activity (for details see text).

after cannulation of the thoracic duct using the method described by Gowans & Knight (1964). The TDL was collected overnight at room temperature into a sterile flask containing 5 ml PBS to which was added 20 U/ml heparin, 2000 U/ml penicillin and 2000 μg/ml streptomycin. The cells were washed three times in supplemented PBS and resuspended to 50×10^6 cells/ml.

With the exception of spleen cells, all suspensions were enriched for T cells using nylon wool columns (Julius, Simpson & Herzenberg, 1973) after which they were resuspended in culture medium and adjusted to 10×10^6 cells/ml.

Assay of helper cell activity

The assay used to determine helper activity of cells in lymphoid tissues from primed rats is shown in Fig. 1. Rats were primed with the carrier protein KLH via one of several different routes and cells from PP, MLN, PLN or TDL (enriched with respect to T cells) assyed for their ability to help DNP-primed B cells (splenic lymphocytes obtained from rats primed with DNP-BGG) to respond to DNP-KLH *in vitro* as described below. KLH-primed T cells were cultured with DNP-primed spleen cells for 5 days, and IgA, IgM and IgG anti-DNP antibody-producing cells enumerated by the Cunningham & Szenberg (1968) modification of the plaque-forming cell (PFC) assay.

Cultures

KLH-primed T cells were cultured with DNP-primed spleen cells (the B-cell source) at ratios of 1:1 or 2:1 ($0{\cdot}5 \times 10^5$ T and $0{\cdot}5 \times 10^5$ spleen cells or $0{\cdot}66 \times 10^5$ T and $0{\cdot}33 \times 10^5$ spleen cells to give a total of 1×10^6 cells per culture) in a volume of 0·2 ml with or without DNP-KLH (0·1 μg/ml). In addition, the KLH-primed T cells and the spleen cells were each cultured alone in the presence of DNP-KLH to assess background numbers of PFC. All cultures were set up in triplicate in Linbro 96-well flat-bottomed tissue culture plates. The culture medium was RPMI-1640 (Flow Laboratories, Sydney, Australia) containing 5% FCS, 5×10^{-5} M 2-mercaptoethanol, penicillin 100 U/ml, streptomycin 100 μg/ml, fungizone 0·25 μg/ml, L-glutamine 2 mM (Flow Laboratories) and Hepes 10 mM. The cultures were fed daily with 25 μl of a nutritive cocktail (Mishell & Dutton, 1967). On Day 5, the cells were harvested, washed twice and assayed to enumerate DNP-specific PFC.

Enumeration of DNP-specific antibody-producing cells

The number of IgA, IgM- and IgG-specific anti-DNP antibody-producing cells in each culture was determined by the PFC method of Cunningham & Szenberg (1968). IgM PFC were detected by the ability of IgM-specific anti-DNP-producing B cells to lyse TNP-coated SRBC [light modification as described by Henry (1980)] in the presence of guinea-pig complement. IgG and IgA were detected by the addition of monospecific rabbit anti-rat IgG (a gift from Dr A. W. Cripps, Hunter Immunology Unit, Royal Newcastle Hospital) and sheep anti-rat IgA (Capell Laboratories, West Chester, PA), respectively.

The helper cell activity of the lymphoid organs of KLH-primed rats is assumed to be proportional to the capacity to help DNP-primed B cells to respond to DNP-KLH *in vitro* and is expressed as the number of PFC per 10^6 spleen cells cultured. It should be noted that this is a measure of *net* T cell help, not an absolute measure of the number of T helper cells, as the different organs may contain different amounts of suppressor factors or of suppressor T cells. In each experiment, cells were pooled from each organ from two rats, and data from at least two or more experiments were used. The PFC arising in the absence of antigen for each culture was subtracted prior to calculation of responses.

The number of PFC per 10^6 spleen cells cultured was then calculated from the following equations, which correct for any background effects due to PFC occurring in spleen cells alone:

$$\text{For 1:1 ratio, PFC} = (2a-(b+c))4$$

$$\text{For 2:1 ratio, PFC} = (3d-(b+2c))4$$

where a = PFC in 1:1 mix of T cells and spleen cells, b = PFC in spleen alone, c = PFC in 2:1 mix of T cells and spleen cells (all data corrected for PFC occurring in cultures without antigen).

The mean PFC was then calculated from the pooled triplicates of the combined experiments.

RESULTS

Antigen specificity of KLH-primed helper cells

The MLN were removed from rats immunized by the IP+ID regime with either OVA or KLH or from unimmunized rats (two animals per group) and tested for their ability to help DNP-BGG-primed B cells to respond to TNP-OVA or DNP-KLH *in vitro* (Fig. 2). MLN from KLH-primed animals provided help for a response to DNP-KLH but not a response to TNP-OVA. The MLN from animals immunized with OVA provided help for a response to TNP-OVA but not to DNP-KLH. MLN from unimmunized animals could not provide help for the response to either DNP-KLH or TNP-OVA. This experiment established that the carrier-primed cells provide antigen-specific help.

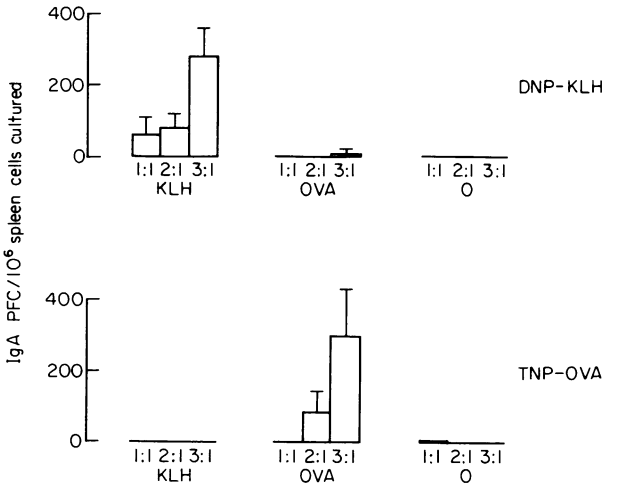

Figure 2. Antigen specificity of the IgA helper cell response. The capacity of MLN cells to provide help for an anti-DNP response among hapten-primed spleen cells cultured with either DNP-KLH or TNP-OVA was measured following IP+ID immunization of MLN donor rats with either KLH or OVA. The helper cell response was measured at 1:1, 2:1 and 3:1 ratios of MLN:spleen cells. The carrier antigen used for immunization of MLN donors is shown under each set of histograms, and the antigen with which the cells were cultured is shown to the right of each graph. Data represent means of triplicate cultures from two rats in each experiment and vertical bars represent standard errors.

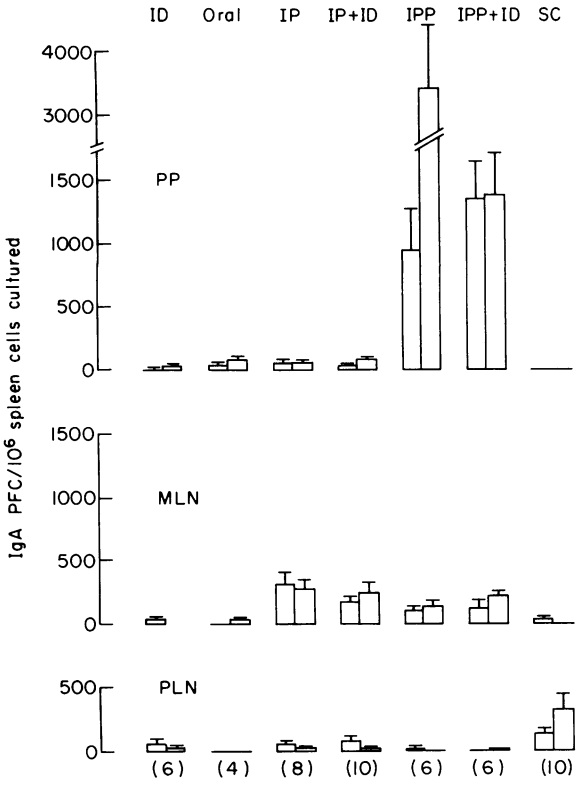

Figure 3. The effect of immunization route on the IgA helper cell response. For each immunization route, the capacity of cells from KLH-primed rats to help spleen cells from hapten-primed rats mount an IgA-specific anti-DNP response to DNP-KLH *in vitro* is shown at two helper cell : spleen cell ratios. For each immunization route, the left histogram is for a 1:1 ratio and the right is for a 2:1 ratio. Values are means of triplicate cultures from the number of rats shown in parentheses and vertical bars represent standard errors.

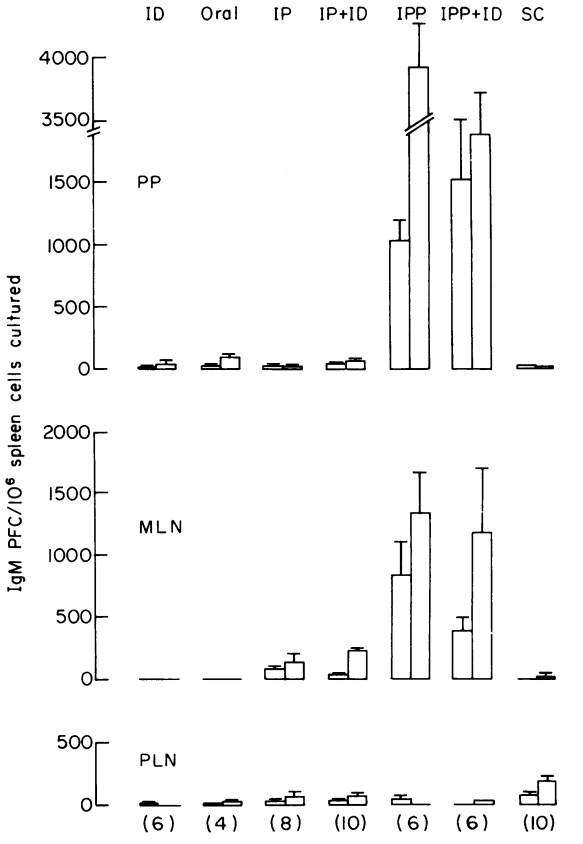

Figure 4. The effect of immunization route on the IgM helper cell response. For each immunization route, the capacity of cells from KLH-primed rats to help spleen cells from hapten-primed rats mount an IgM-specific anti-DNP response to DNP-KLH *in vitro* is shown at two helper cell : spleen cell ratios. For each immunization route, the left histogram is for a 1:1 ratio and the right is for a 2:1 ratio. Values are means of triplicate cultures from the number of rats shown in parentheses and vertical bars represent standard errors.

Effect of immunization route on helper cell response

Various immunization routes were studied for their effectiveness in producing helper cells in PP, MLN and PLN. The capacity of T cells from these tissues to provide help for IgA, IgM and IgG anti-DNP responses among BGG-primed cells after coculture with DNP-KLH are shown in Figs 3, 4 and 5.

Prolonged oral administration or a single ID injection of KLH gave little or no response in any of the tissues studied, indicating that for this soluble antigen oral stimulation is insufficient to produce a detectable helper cell response. Both IP and IP + ID immunizations stimulated the appearance of cells in MLN, which provided help for an IgA and IgM response, but the response in PP and PLN was negligible. An ID challenge following IP immunization had no obvious effect on the IgA or IgM response, indicating that the IP component of this immunization regime is sufficient to prime helper cells in MLN for responses of these isotypes. It is of interest to recall that the subsequent ID challenge is necessary in order to obtain a substantial IgA B-cell response in the gut lamina propria after IP priming (Pierce & Gowans, 1975). No IgG help was detected in MLN after IP immunization, but IP + ID immunization did elicit an IgG helper response.

IPP immunization was found to be the most effective route for the induction of a helper response from GALT. Following this immunization regime, a large helper response for all isotypes was found in PP, a smaller response in MLN and virtually no response in PLN, indicating that the helper response following IPP immunization is restricted to GALT tissues. Again, an ID challenge following IPP immunization did not enhance the helper cell response but in some cultures resulted in a reduced response, particularly for IgG. Systemic immunization via the SC route stimulated a significant helper response for all isotypes in PLN, with little or no response in PP and MLN.

Kinetics of the helper response following IPP immunization

Since IPP immunization gave the best helper response from GALT for all isotypes, this route was chosen for subsequent kinetic studies. Thus, PP, MLN, TDL and PLN were examined for helper cell content at various times following immunization via the IPP route (Figs 6, 7 and 8). The helper response in PP was detectable for all isotypes at Day 2, declined on Day 3, then steadily climbed to a peak at 2 weeks, after which it again declined. A similar pattern but of lower magnitude was observed in MLN and TDL, although, IgA-specific help was not

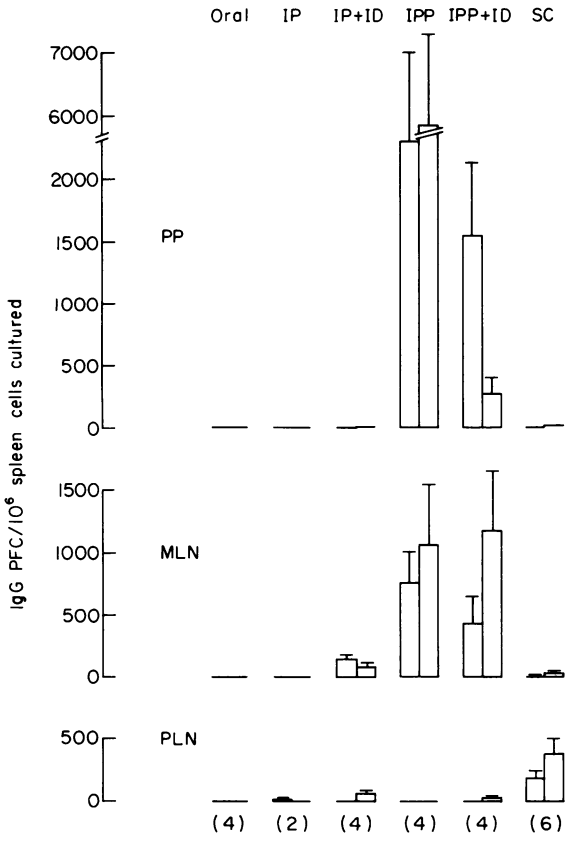

Figure 5. The effect of immunization route on the IgG helper cell response. For each immunization route, the capacity of cells from KLH-primed rats to help spleen cells from hapten-primed rats mount an IgG-specific anti-DNP response to DNP-KLH *in vitro* is shown at two helper cell : spleen cell ratios. For each immunization route, the left histogram is for a 1:1 ratio and the right is for a 2:1 ratio. Values are means of triplicate cultures from the number of rats shown in parentheses and vertical bars represent standard errors.

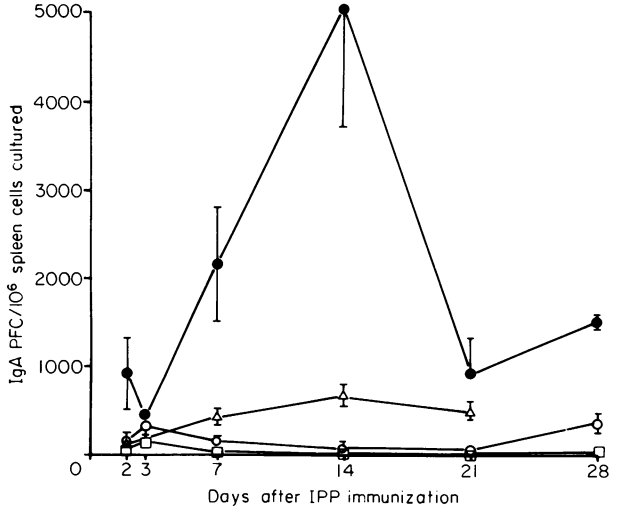

Figure 6. Kinetics of the IgA-specific helper response in PP (●——●), MLN (○——○), TDL (△——△), and PLN (□——□) following IPP immunization. Values represent means of triplicate cultures from four rats for PP, MLN and PLN and from three rats for TDL for each time point. Vertical bars represent standard errors.

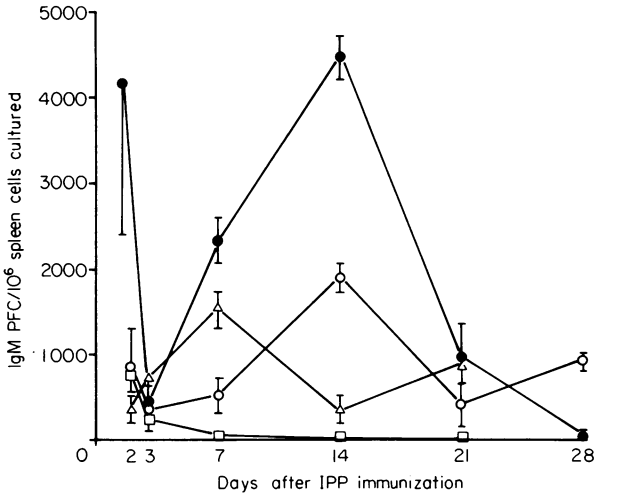

Figure 7. Kinetics of the IgM-specific helper response in PP (●——●), MLN (○——○), TDL (△——△) and PLN (□——□) following IPP immunization. Values represent means of triplicate cultures from four rats for PP, MLN and PLN and from three rats for TDL for each time point. Vertical bars represent standard errors.

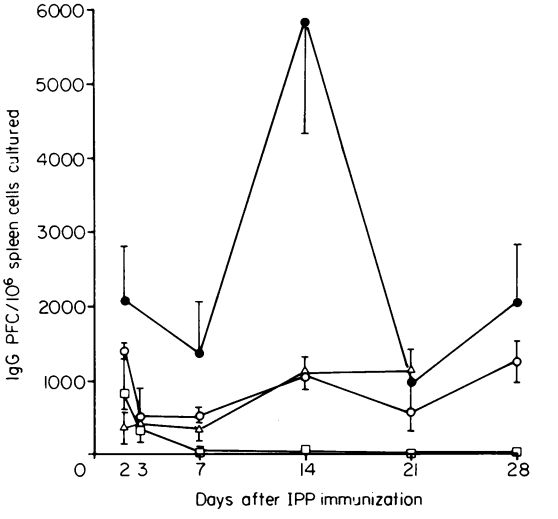

Figure 8. Kinetics of the IgG-specific helper response in PP (●——●), MLN (○——○), TDL (△——△) and PLN (□——□) following IPP immunization. Values represent means of triplicate cultures from four rats for PP, MLN and PLN and from three rats for TDL for each time point. Vertical bars represent standard errors.

substantial in MLN after Day 3. Apart from small levels of IgM- and IgG-specific help at early times after immunization, virtually no helper activity could be detected in PLN at any of the times studied. The presence of helper activity in TDL demonstrates that helper cells generated by GALT immunization can migrate, and probably originate in PP and/or MLN. The helper activity in MLN may represent cells migrating from PP, or they may have arisen in the MLN.

DISCUSSION

This study has demonstrated that antigen-specific helper cells

for IgA responses appear in GALT of rats after intestinal immunization. KLH administered directly to PP stimulated a strong KLH-specific helper cell response in PP and MLN for IgA, and also for IgM and IgG isotypes. At the same time, little or no response could be found in PLN. The substantial IgG helper response observed in GALT could have been influenced by the soluble nature of the carrier antigen used, since particulate antigens are usually more efficient at stimulating GALT IgA responses (Arny *et al.*, 1984). Alternatively, the choice of spleen as a B-cell source could have created a bias towards an IgG response, although others have obtained vigorous IgA responses in splenic B-cell cultures with sheep erythrocyte-specific T_{Ha} cells (Kiyono *et al.*, 1982), and, when in some of our experiments PP B cells were used, there was no difference in the isotype distribution of the response compared with splenic B cells (data not shown). It should also be noted that it is not valid to compare the magnitude of the helper response for different isotypes within each tissue studied because of potential differences in the relative efficiency with which each of the developing antisera perform in the PFC assay; however, comparisons between different tissues within isotypes are meaningful.

The failure of KLH administered orally or by ID injection to stimulate help is in agreement with observations of Arny *et al.* (1984) and Elson & Ealding (1984), and could be due to (i) inadequate uptake of this soluble antigen across the gut wall, (ii) an insufficient concentration of antigen dose, or (iii) an active stimulation of suppression following oral presentation of antigen. The first is unlikely, since in experiments with the soluble antigens OVA and tetanus toxoid an ID dose has been shown to elicit a strong IgA B-cell response in gut lamina propria following IP priming (Husband, 1982). Antigen handling factors, the concentration of antigen in PP and its duration there, are probably of greater importance, although suppression may be involved since oral administration of SRBC to rats has been shown to generate antigen-specific suppressor cells in PP and MLN (Mattingly & Waksman, 1978). It should be emphasized that the data presented here are for 'net' help present in each tissue, that is the sum of helper and suppressor factors, and no attempt was made to enrich tissues for cells of helper phenotype or to deplete suppressor cells. However, the substantial responses obtained after IPP immunization suggest that the injection of KLH under the serosa of the PP either fails to stimulate suppressor cells or simply provides the antigen in sufficient quantity and for a sufficient length of time to stimulate a greater helper response.

It is of interest in this regard that the addition of cholera toxin to KLH prior to oral administration induces an enhanced IgA-producing B-cell response to KLH in the gut (Elson & Ealding, 1984). This indicates that cholera toxin may alter the way in which antigen is handled, possibly creating a level of antigenic stimulation sufficient to generate a net helper response. This possibility could be verified using the experimental regime described in this report.

It is demonstrated here that IP and IPP administration of antigen prime GALT tissues (in particular PP and MLN) for an IgA-specific helper cell response with or without subsequent ID administration of antigen. It has previously been shown that IP immunization (Pierce & Gowans, 1975) or IPP immunization (Husband & Dunkley, 1985) does not stimulate an IgA B-cell response in rats but primes GALT for an IgA response in the gut lamia propria following subsequent ID challenge. In the data presented here, ID challenge did not appear to augment the helper response following IP priming, although this could have been influenced by timing, in that IP + ID immunized rats were killed at 3 weeks after IP priming to ensure that sufficient time had elpsed for a response to ID challenge whereas IP immunized rats were killed at 2 weeks. Nevertheless, these data suggest that IP or IPP priming generates a population of helper cells (presumably T cells of the helper/inducer phenotype), which is probably a prerequisite for a B-cell response to subsequent lumenal administration of antigen. The mechanism by which IP administration of antigen primes GALT is unclear. The antigen presumably directly stimulates MLN and PP via the serosal surface, which is exposed to antigen administered into the peritoneal cavity, although this route of immunization was much less efficient at generating a PP response than direct IPP immunization by subserosal injection.

Following IPP immunization, IgA-specific helper cells were detected in PP as early as 2 days, rising to a peak at 2 weeks and then declining, and similar kinetics were observed in MLN and TDL. The presence of IgA helper cells in TDL demonstrates their ability to migrate from GALT, and further studies are in progress to determine the precise origin of these cells and whether they disseminate to the gut lamina propria and other mucosal sites after entering the circulation. The antigen-dependent proliferation of IgA-specific antibody-containing cells after extravasation into the gut lamina propria (Husband & Gowans, 1978; Husband, 1982) could therefore be under the control of locally situated T_{Ha} cells originating from GALT in response to a priming dose of antigen and reaching the lamina propria via TDL and blood circulation.

The experiments described in this paper demonstrate that in rats, as in mice, a population of antigen-specific cells with T_{Ha} activity appears in GALT after intestinal immunization, that PP are an enriched source of these cells if apporopriately immunized, and that these cells have the capacity to migrate via TDL. The possibility that GALT-derived T_{Ha} cells entering the circulation may populate mucosal sites other than the intestine is an extension to current concepts of a common mucosal immune system that requires further investigation.

ACKNOWLEDGMENT

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