Nodular lymphoid hyperplasia of the bowel in primary hypogammaglobulinaemia: study of *in vivo* and *in vitro* lymphocyte function

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SUMMARY   *In vitro* and *in vivo* lymphocyte function was studied in six patients with primary hypogammaglobulinaemia and nodular lymphoid hyperplasia (NLH) of the bowel. Lymphocyte transformation, numbers of circulating T and B lymphocytes, and delayed hypersensitivity skin tests did not significantly differ when compared with hypogammaglobulinaemic patients without NLH. However, patients with NLH had higher jejunal juice IgM concentrations and a tendency to higher serum IgM concentrations than those without NLH. The morphological features of NLH are similar to the germinal centres of lymph nodes but more closely resemble the follicle zone of Peyer's patches. These findings suggest that NLH represents a local immune response to antigens originating in the gut lumen.

Nodular lymphoid hyperplasia (NLH) of the bowel is a condition virtually confined to patients with primary immunodeficiency. It is extremely rare in children and is generally a complication affecting adults with primary common variable late onset hypogammaglobulinaemia. Six patients were found to have NLH in the course of screening for gastrointestinal complications and we have analysed *in vitro* and *in vivo* lymphocyte function in these patients to establish whether they have any characteristics in common. In addition, we have attempted to identify the cell types within the nodules.

Methods

SUBJECTS

Thirty-four patients with primary hypogammaglobulinaemia were screened for NLH. They were selected on the basis of their willingness to be inpatients and there was no tendency for patients with gastrointestinal complaints to be referred. Their ages at the time of investigation ranged from 7 to 63 years (mean 33 years). Seventeen patients were receiving regular weekly intramuscular gammaglobulin at a dose of 25-50 mg/kg body weight.

Twenty-eight patients had grossly depressed levels of serum IgG—that is, <50 IU/ml—mean = 29 IU/ml) and only six patients had levels above 50 IU/ml. Serum IgA concentrations were unrecordable in 29 patients—that is, <3·5 IU/ml—and only one patient had a level within the normal limits. Twenty-eight patients had serum IgM concentrations below 47 IU/ml (mean = 12). The remaining six patients had normal serum IgM concentrations. Our normal adult laboratory levels expressed as geometric means with the 95% range of results are as follows: IgG 122 (80-185), IgA 141 (51-389), IgM 136 (59-308) IU/ml. Six patients developed recurrent infections before the age of 2 years, 10 patients between the age of 2 years and 16 years, and 18 patients after the age of 16 years. Three patients had sex-linked hypogammaglobulinaemia with an affected male sibling and the rest had variable (late onset or acquired) hypogammaglobulinaemia.

TECHNIQUES

Gastrointestinal assessment
Patients were given a diet containing 100 g of fat daily and, after two days, stools were collected for a further five days for fat estimation. At least two

fresh stools were examined for parasites. A standard xylose absorption test was performed after a 25 g oral dose of xylose. Each patient had a barium meal and follow through examination with particular attention being paid to identifying NLH.

Patients with evidence of malabsorption—that is, faecal fat >5 g/24 h and/or a 5 h urinary xylose excretion <33 mmol—or NLH on barium meal were further investigated by jejunal biopsy. Rectal biopsies were also obtained from most cases. When possible, jejunal juice was collected and examined for the presence of parasites.

Immunological studies

Immunoglobulin concentrations were measured by a modified Mancini method. A sensitive technique based on the method of Lundkvist and Ceska (1972) was used for unconcentrated saliva and jejunal juice. Two per cent Carbowax 6000 was incorporated into a 3% agar (Ionagar No. 2, Oxoid) and antiserum gell. Class specific sheep antihuman serum (Burroughs Wellcome) was used at the following approximate concentrations in the gell: anti IgG 1:300, anti IgA 1:150, anti IgM 1:120. The wells were filled twice with the test sample and then again with 50% Carbowax 200 in saline. This enabled us to measure concentrations of immunoglobulin down to 0.2 mg/100 ml. Unstimulated saliva was collected after the mouth was examined to exclude periodontal inflammation, by asking the patients to expectorate into a container. The saliva was then immediately centrifuged at 4000 × g for 15 minutes and stored at −20° until tested. The day to day variablity of salivary immunoglobulin concentrations in normal persons was about 20%. Jejunal juice was collected by small bowel intubation and approximately 25 μl Trasylol (Ciba) added to 1 ml and the whole kept at −20°C until tested. A standard serum, previously estimated against the standard MRC serum 67/99, was used for all the measurements of IgG, IgA, and IgM. The results for IgA concentrations in secretions were adjusted according to the recommendations of Hobbs (1971).

Serum IgE was measured in some patients with a method previously described by McLaughlan, *et al.* (1971). Serum IgD was measured by a modified Mancini technique using swine antihuman IgD (Nordic Diagnostics) at a final dilution in the agar gell of 1:80 and filling the wells twice with undiluted test serum. The MRC standard serum for IgD, 67/37, was used in this assay.

In vitro lymphocyte studies were performed on the cells from 13 patients. Cells were obtained from heparinised venous blood after separation on a Ficoll-triosil density gradient. Lymphocyte transformation was performed using a microtray method

(Webster and Asherson, 1974). Lymphocytes were stimulated with purified phytohaemagglutinin (PHA-Burroughs Wellcome) at 0.1, 0.5, 5, and 50 μg/ml final concentration; Concanavalin A (Pharmacia) at 1, 10, 50, and 100 μg/ml and pokeweed mitogen (Grand Island Biologicals) at approximately 35 and 70 μg protein/ml. H^3 thymidine (1 uCi) (specific activity 50 mCi/mmol) was added to each culture at 48 hours and the cells harvested 16 hours later. A mixed lymphocyte reaction with the lymphoid cell line CLA 4 (Steel *et al.*, 1973) was performed using 50 × 10^4 lymphocytes and 8 × 10^6 irradiated (5000r) CLA 4 cells per culture. H^3 thymidine was added at 152 hours and the cells harvested at 168 hours. Peripheral blood B lymphocytes were identified by surface immunoglobulin staining with fluoresceinated goat anti-human globulin (Behringwerke). Cells with surface receptors for altered C3 were identified using the method of Nussenzweig *et al.* (1971). Peripheral blood T cells were recognised by their ability to form spontaneous rosettes with sheep red blood cells (Wybran *et al.*, 1973). The following delayed hypersensitivity skin tests were performed: intradermal injections of 0.1 ml of 1% *Candida albicans* solution (Bencard) and a Heaf test. Contact sensitivity to dinitrochlorobenzene was tested using a 5% sensitising dose in acetone followed by challenge with 0.1% 7 days later.

Immunofluorescent studies were performed on the jejunal biopsies from the six patients with NLH, four of which contained the actual nodules. The direct immunofluorescent technique (Shiner and Ballard, 1973) was employed using acetone fixation after snap freezing the tissue in all but one patient in whom the biopsy was fixed in 2.5% formaldehyde before freezing according to the method of Savilahti (1972). Fluorescein isothiocyanate-conjugated antisera to IgG (Wellcome), and IgA (Travenol Laboratories) were used at dilutions between 1/20 and 1/60. Sections were studied blindly and in random order on a Leitz Orthoplan microscope. The number of plasma cells seen in the jejunal mucosa was graded on a scale of 1-4, with normal controls being 1 for IgG, 2 for IgM, and 4 for IgA.

Paraffin embedded biopsy specimens were also stained using peroxidase labelled antisera. These sections had been previously fixed in 10% formalin in saline for a minimum of four hours and dehydrated in alcohols before paraffin embedding; 4 μ sections were brought to water and, after washing with buffer, exposed to specific class rabbit antihuman immunoglobulin (Dakopatts). After further washing, peroxidase labelled sheep antirabbit antisera was added (Pasteur Institute). After washing in buffer, the peroxidase was developed by a standard DAB method.

Table 1   *Relationship between serum, salivary, and jejunal juice immunoglobulins and cells with intracytoplasmic immunoglobulin in jejunal biopsies of patients with and without biopsy and radiographic evidence of nodular lymphoid hyperplasia*

Patient	Age (yr)	IgG IU/ml			Cells in lamina propria		IgA IU/ml			Cells in lamina propria		IgM IU/ml			Cells in lamina propria		Cells within nodule	
		Serum	Saliva	Jej. juice	f	p	Serum	Saliva	Jej. juice	f	p	Serum	Saliva	Jej. juice	f	p	f	p
Patients with NLH:																		
C.B.*	63	70·3	0·1	0	0	0	<2	0	0	0	0	26	0	6	+	0	Int.IgM	0
R.F.	30	<6·2	0	ND	0	0	6	0·6	ND	++	0	<2	0	ND	0	0	0	0
J.W.	40	17·4	0·04	0	+	0	<2	0·5	0	0	0	66	0	8	+	0	NS	NS
M.H.*	40	31·1	0·04	0	0	0	<2	0	0	0	0	45	3	18	+++	+	Int.IgM	0
P.M.*	45	79·6	0	0·2	+	0	<2	0	0·9	0	0	15	0	12	0	+	Int.IgM	0
W.F.*	20	36·1	0	0	0	ND	<2	0	0	0	ND	0	0	4	+++	ND	NS	NS
Patients without NLH:																		
C.H.	25	6	2	3	0	0	<2	0·2	0	0	0	<2	0	4·5	0	0		
C.C.	38	<1	0	0	0	0	<2	0	0	0	0	<2	0	0	0	0		
S.C.	38	17	0·3	0	0	0	<2	0	0	0	0	<2	0	0	0	0		
M.H.	38	6	0	2	ND		15	0	ND	ND		12	0	0	ND			
V.P.	33	15	ND	0	++	0	<2	ND	0	++	0	0	ND	0	++	0		
E.B.*	54	39	ND	0	0	0	<2	ND	0	0	0	38	ND	0	0	0		
L.T.*	64	41	0	0·8	++	0	<2	0	0	+	0	<2	0	0	0	0		

ND: not done. NS: no nodules available for study. f: fluorescein labelled antisera. Int. IgM: appearance of interstitial fluorescent staining. p: persoxidase labelled antisera.
*Treated with weekly gammaglobulin injections.
The figure 0 means undetectable—that is, 0·04 IU/ml for IgG, 0·2 IU/ml for IgA, 0·9 IU/ml for IgM.

Table 2   *In vivo and in vitro lymphocyte studies in patients with and without evidence of nodular lymphoid hyperplasia on barium follow through*

		Patients with NLH (n = 6)	Patients without NLH (n = 7)	Normal controls (n = 22)
Lymphocyte transformation to: (expressed as counts/min/25 × 10⁴ cells)	PHA	3059 (1033-9063)	3368 (1223-9274)	7029 (4575-10800)
	Con A	2067 (1395-3063)		5090 (3038-8527)
	PWM	604 (325-1122)		1306 (769-2219)
	CLA 4 cells	2120 (708-7009)	4342 (1438-13108)	2984 (5608-1588) 20*
Peripheral blood lymphocyte count % circulating	T cells (E rosettes)	63 (54-74)	56 (40-79)	60 (47-77)
	B cells (surface Ig)	11 (7-19)	9 (5-15) 6*	15 (11-20)
	(EAC rosettes)	14 (9-21)	16 (11-24) 6*	16 (13-20)
Positive delayed hypersensitivity skin tests	Candida	4	1 6*	
	Heaf	2	1 6*	
	DNCB	2	1 6*	

Differences between the two groups with and without NLH did not reach significance (P > 0·05). Results are shown as geometric mean with the ISD range in parentheses.
Numbers of patients and controls studied are stated on top of columns except where indicated by asterisk.
Abbreviations: PHA: Pnytohaemagglutinin: Con A: Concanavalin A. PWM: pokeweed mitogen. The optimal mitogen dose response is recorded.

For electron-microscopy, the jejunal mucosa was immediately fixed in a 3% glutaraldehyde-cacodylate solution, followed by post-fixation in 1% phosphate buffered osmium, dehydration in graded alcohols, and embedded in epoxy resin (Epon 812) (Shiner, 1967). Nodules were identified with the electron microscope in only two biopsies.

Results

A positive diagnosis of NLH was considered only if nodules were found in the jejunal biopsy. Four patients had radiographic evidence of NLH but were excluded from the study because the biopsy was negative. Table 1 compares the immunoglobulin concentrations in serum, saliva, jejunal juice, and the class of cytoplasmic immunoglobulin found in lymphocytes within the jejunal lamina propria in patients with NLH and in patients without radiographic or biopsy evidence of NLH. All five patients tested with NLH had significant amounts of IgM in jejunal secretions, although only one had detectable salivary IgM. In contrast, only one of the patients without NLH had detectable jejunal juice IgM. There was also a tendency for the serum IgM concentrations to be higher in patients with NLH. IgA was not detected in the serum or secretions of most patients. IgE and IgD levels were measured in

five of the patients with NLH and in four (IgD) and seven (IgE) of those without NLH. There were no differences between the two groups. Table 2 compares the results of *in vitro* lymphocyte function tests and delayed hypersensitivity skin tests in the six patients with and in the seven patients without NLH. There was no significant (P > 0·05) difference between the two groups.

Immunofluorescent studies were performed on nodules in biopsy specimens from three patients. IgM, but not IgA or IgG, fluorescence was seen in all three, being most marked in patient PM (Fig. 1), but it was doubtful whether this was intracellular or interstitial staining. The latter interpretation was supported by the absence of immunoglobulin containing cells in nodules from four patients stained with peroxidase labelled antisera (Figs. 2b, 3b, and Table 1).

MORPHOLOGICAL FEATURES
Dissecting microscopy of the jejunal biopsies of the six patients with NLH showed mucosal leaves and convoluted ridges; finger villi were uncommon. A gross nodularity was not apparent.

In paraffin sections, the nodules were composed of germinal centres with a sourrounding mantle of lymphocytes (Figs. 2a, 3a). This was particularly evident in a rectal biopsy taken from patient MH who had gross NLH affecting both the small and large bowel (Fig. 3a). In individual sections, germinal centres were sometimes lacking, the nodules showing only aggregates of mature lymphocytes, but sections at levels through the biopsy revealed the characteristic structure. The nodules were present in the lamina propria with partial or minor disturbance of the villous pattern. In one case there was extension of a nodule through the muscularis, splitting the muscle fibres and extending into the submucosa. In the centre of the nodules, pale histiocytic and macrophage cells were seen. Occasional 'tingible macrophages' were noted, suggesting some cell death. There were also moderate numbers of small lymphocytes which merged with the surrounding cuff of lymphocytes. The histological features of the nodules were similar to those seen in the germinal centres of reactive lymph nodes.

The lamina propria and villi away from the nodules appeared normal. There was no excess infiltration with inflammatory cells. Plasma cells, confirmed by fluorescein labelled antisera as containing immunoglobulin, were present in normal numbers in only two patients and were very scanty in another two patients. Scattered pyroninophilic mononuclear cells were present in all six patients.

ELECTRONMICROSCOPY
Nodules from two patients were examined. Two main features were observed: (1) the presence of a fibrous capsule in patient MH which contained collagen fibres (Fig. 4); (2) the centres of the nodules were necrotic and contained cellular debris and altered nuclei of unidentifiable cell type (Fig. 5). In the biopsy of MH, many plasma cells of normal appearance could be seen in the mucosal connective tissue surrounding the nodule but within the nodule there were unusual cells containing phagocytosed material and a considerable amount of endoplasmic reticulum (Fig. 6). Small lymphocytes
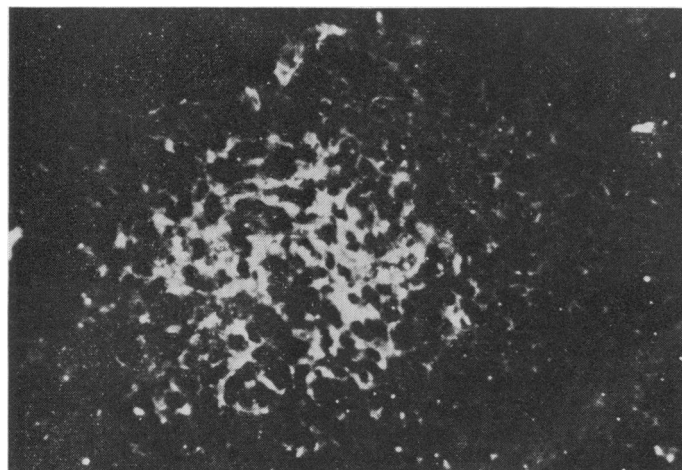

Fig. 1   *Nodule stained with fluoresceinated anti IgM serum from P.M; some of the fluorescence is probably intracytoplasmic. Original magnification, × 150.*

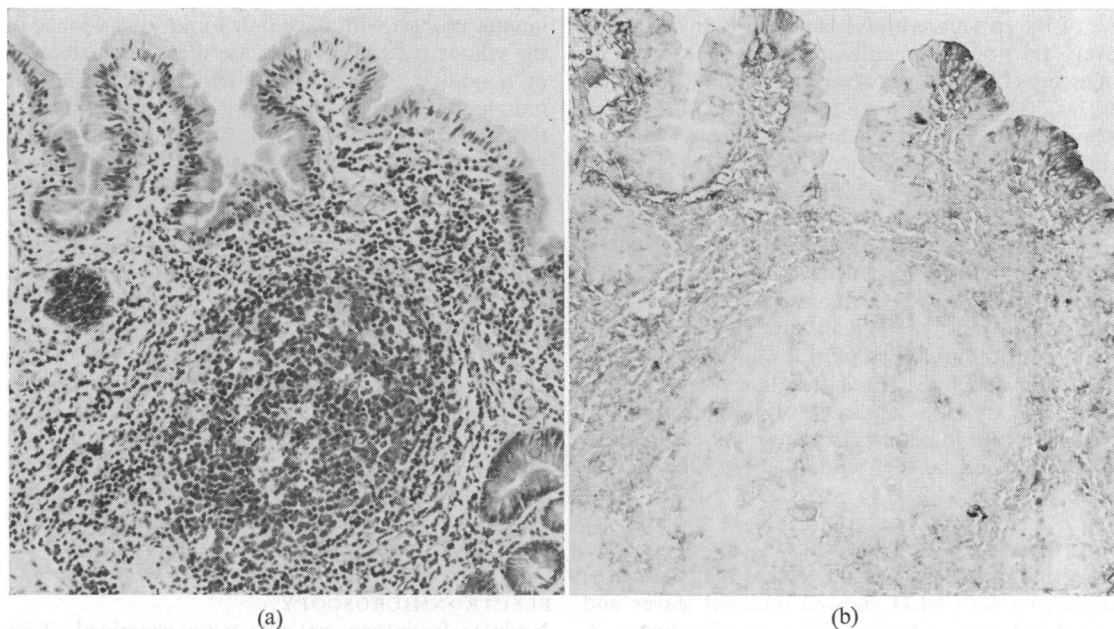(a)   (b)

Fig. 2   (a) Section of nodule in jejunal biopsy from C.B. showing appearance similar to the germinal centres of lymph nodes. Haematoxylin and eosin. Original magnification, × 135. (b) Next section stained for immunoglobulin with peroxidase labelled antisera. No immunoglobulin containing cells are seen within the nodule.

could be seen outside and immediately within the nodule of JW but this was the only cell type to be definitely identified.

Discussion

This study shows that NLH occurs in at least 19% of patients with late onset variable hypogamma-globulinaemia. This is probably an underestimate, as barium follow-through examination may not reveal small nodules. Only two patients had giardiasis (Table 3) which is at variance with the findings of Ajdukiewicz (1972) who, in reviewing the literature, found that 15 of 21 patients with NLH had giardiasis. However, many of these cases were from North America where giardiasis accounts for most of the malabsorption in patients with hypogammaglobulin-aemia (Ament et al., 1973). The experience in England is different, in that giardiasis accounted for only about one-third of cases with malabsorption in our series. This dichotomy is probably related to environmental differences but indicates that there is likely to be no direct aetiological relationship between giardiasis and NLH.

NLH is probably a benign disorder, although there has been a report of the condition being associated with a gut lymphoma in a patient with apparently normal immunity (Kahn and Novis 1974). Weekly

gammaglobulin injections do not appear to influence the condition, as three of our patients were known to have had NLH for more than three years while receiving such therapy. A few patients with variable hypogammaglobulinaemia develop a marked generalised lymphadenopathy, the lymph nodes becoming packed with apparently normal germinal centres. We have observed such a patient in whom there was no evidence of NLH at necropsy; a finding which suggests that these two conditions are not aetiologically linked.

The morphology of NLH is clearly similar to the germinal centres of reactive lymph nodes and the absence of cells containing immunoglobulin within the nodules is also compatible with this comparison. However, although lymph node biopsies were not taken in these patients, failure of lymph node germinal centre formation is considered to be one of the hallmarks of hypogammaglobulin-aemia. Nevertheless, there are differences in the detailed morphology of NLH and lymph node germinal centres in that the former are often not surrounded by a dense halo of small lymphocytes and there are usually not very many macrophages containing tingible bodies.

The electronmicroscopic studies in patient MH showed the presence of unusual phagocytic cells containing endoplasmic reticulum. The phago-
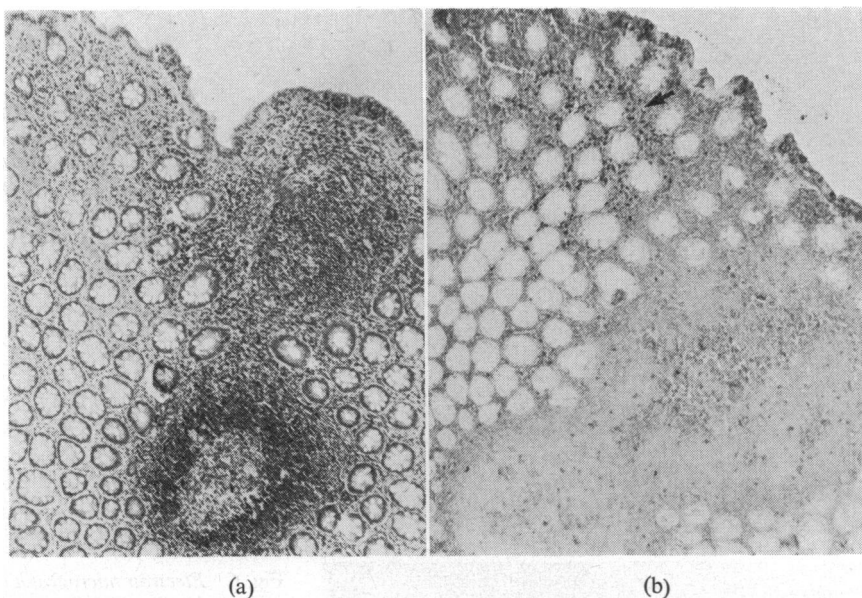
(a)

(b)

Fig. 3   (a) *Horizontal section through rectal biopsy from patient MH who also had extensive NLH of the large bowel. Haemotoxylin and eosin. Original magnification, × 60. (b) Next section (×75) stained with peroxidase labelled anti-IgM serum showing no immunoglobulin containing cells within the nodules. However, IgM containing plasma cells can be seen elsewhere in the lamina propria (arrowed). (c) Different section of rectal biopsy from patient MH showing a lymphoid 'organ' with very similar morphology to the Peyer's patch. Note dome (D) and follicular zones (F) which appear to be separated. Haematoxylin and eosin. Original magnification × 60.*
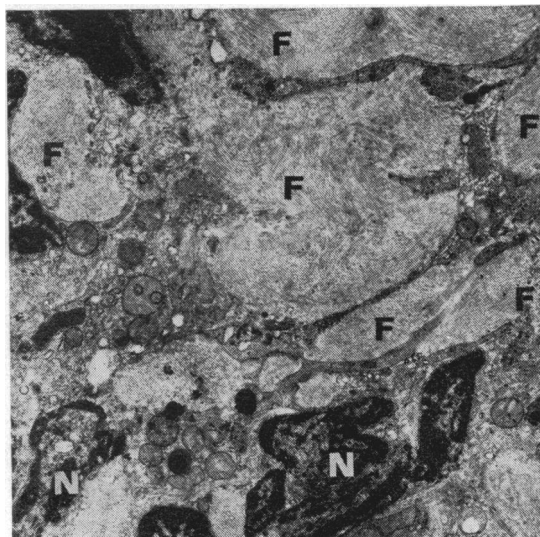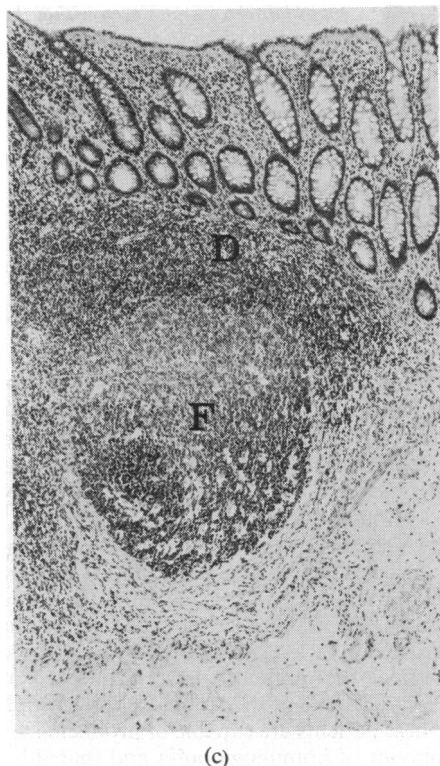

(c)

Fig. 4   *Electron micrograph of nodule from patient MH. Thick bundles of fibres (F) are seen at the periphery of the nodule. Many of the fibres show the characteristic periodic cross-striation of collagen. Necrotic nuclei (N) are also seen. × 5000 (approximately).*

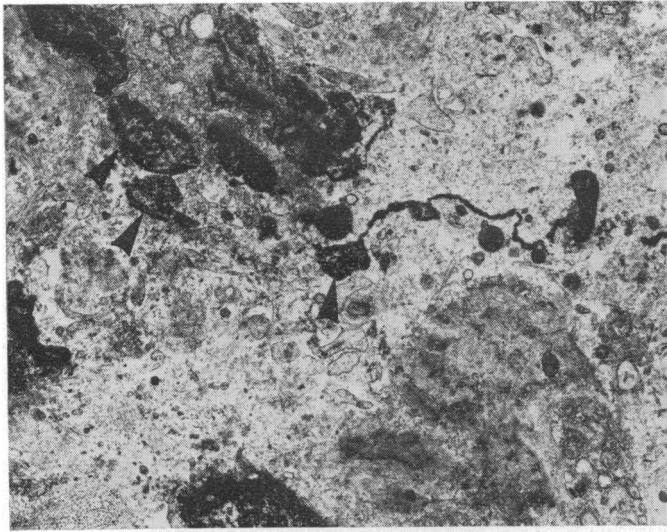Fig. 5 *Electron micrograph of centre of nodule from patient MH. Only necrotic material and nuclei (arrow) can be identified.* × 5000 *(approximately).*
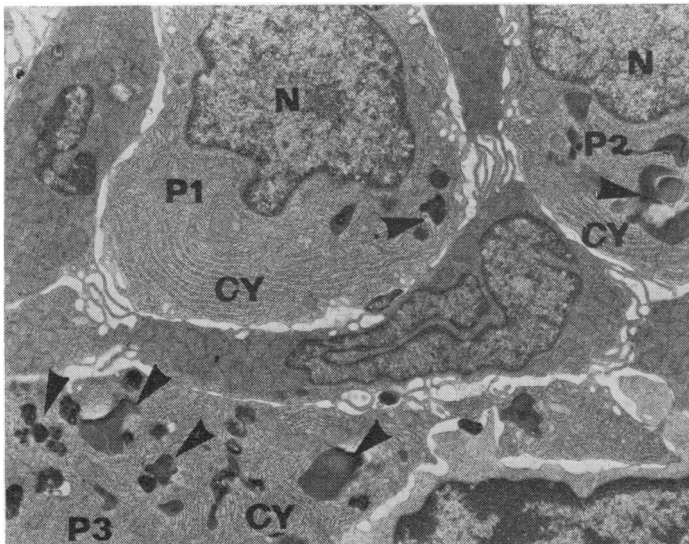

Fig. 6 *Electron micrograph of several abnormal cells (P1,2,3) near the periphery of the nodule from patient MH. The nuclei (N) of these cells have irregular contours and show a chromatin arrangement resembling that of macrophages. Numerous vacuoles containing phagocytosed material (arrows) are seen within the cytoplasm which is also packed with cysternae (CY) lined by rough endoplasmic reticulum. These cysternae, normally found in large numbers in the cytoplasm of plasma cells, appear to be empty and are arranged in parallel fashion to each other.* × 10 000 *(approximately).*

cytosed material was probably either partly digested bacteria or nuclear debris. These appearances and the apparent absence of dendritic macrophages are similar to those found in the follicular zone of Peyer's patches (Watanabe and Tashiro, 1971). The finding of a fibrous capsule around a nodule from MH is not consistent with either a germinal centre or Peyer's patch anology and was probably a 'chance' finding, as in all the other nodules examined there was no sign of such a capsule.

Ajdukiewicz *et al.* (1972) in their review, found no evidence that patients with NLH were identifiable by certain *in vitro* and *in vivo* immunological tests. Our attempts, using a wider variety of tests, confirm this view (Table 3). However, the optimum time of five to six days for PWM induced lymphocyte transformation was not used in this study, although it is unlikely that longer incubation would have given different results. Jejunal juice IgM concentrations were significantly higher in patients with NLH and the immunofluorescent studies suggested that this IgM was being produced locally by cells in the lamina propria. In some instances, peroxidase labelling techniques failed to confirm the presence of such cells, suggesting that the IgM had originated from the serum. A likely explanation for the dichotomy between the immunofluorescent and peroxidase findings is that some of the lamina propria lymphoid cells in these patients are capable of producing a very small amount of immunoglobulin and that this can

Table 3 *Relationship between giardiasis, diarrhoea, and malabsorption in patients with nodular lymphoid hyperplasia*

Patient	Present age (yr)	Age of onset of recurrent infections (yr)	Giardia		Diarrhoea >3 semi-fluid motions/day	Faecal fat (g/24h) (normal <5)
			Stool	Jej. juice		
C.B.	63	57	Pos	Pos	Yes	10·8
R.F.	30	11	Pos	nd	Yes	2·4
J.W.	40	36	Neg	Neg	Yes	13·2
M.H.	40	27	Neg	Neg	No	5·0
P.M.	45	10	Neg	Neg	Yes	5·4
W.F.	20	1	Neg	Neg	Yes	7·2

be identified only with the more sensitive immuno-fluorescent techniques.

B lymphocytes are known to be important in the development of both the germinal centres of lymph nodes in animals (Brown *et al.*, 1970) and Peyer's patches (Nieuwenhuis and Keuning, 1974). This may explain why NLH has not yet been described in sex-linked 'agammaglobulinaemia' with absent B lymphocytes. The reason why NLH can occur when lymph node germinal centre formation fails may also be explained by the finding that the gut in patients with variable hypogammaglobulinaemia is a relatively privileged site for B cell maturation (Broom *et al.*, 1975).

The available information suggests that NLH represents a local immune response to antigens such as viruses or bacteria. A moderate overgrowth of aerobic and anaerobic bacteria is common in the jejunal juice of patients with primary hypogamma-globulinaemia (Webster, 1976). However, the total number of bacteria in the jejunal juices of most patients studied here did not exceed 10^6/ml and there was no tendency for those with NLH to have higher bacterial counts.

Although similar to lymph node germinal centres, the nodules more closely resemble the follicle zone of Peyer's patches (Fig. 3c, Faulk *et al.*, 1970). The reason why such nodules do not usually occur in healthy adults may reflect an ability to produce appropriate antibody which either neutralises the offending antigen or normally acts to regulate such lymphoid hyperplasia. A temporary failure to produce such antibody may explain the transient occurrence of NLH seen in some apparently 'healthy' children in the terminal ileum or colon (Fieber and Schaefer, 1966: Capitanio and Kirkpatrick, 1970).

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