Histological response and antigen transmission in the lymph nodes of athymic *nu/nu* mice inoculated with Crohn's disease tissue filtrates

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SUMMARY Lymph node histology and antigen transmission in the *nu/nu* mouse in response to animal inoculation with Crohn's disease tissue filtrates were re-evaluated. We found that a hyperplastic lymph node response in *nu/nu* mice occurred with Crohn's disease (CD), ulcerative colitis (UC), or other intestinal disease (OID) tissue inoculations. In addition, antigen transmission to lymph nodes as detected by indirect immunofluorescence using CD sera was observed in all inoculation groups. The immunofluorescent reaction also occurred independently of lymph node histology. Thus, we confirm that CD sera recognize an antigen(s) expressed in lymph nodes of athymic mice inoculated with CD tissue filtrates. The antibody (or antibodies) in CD sera was not specific for this 'CD antigen or antigens', however, as tested in the *nu/nu* mouse system, because the CD sera antibodies also recognised antigens in UC inoculated and OID inoculated animals.

In 1980, Das *et al*¹ first used the athymic T-cell deficient *nu/nu* mouse model in an attempt to identify a disease specific transmissible agent of Crohn's disease (CD). They reported that six of 34 athymic mice injected with CD tissue filtrates developed gross lymphadenopathy, which on histological examination revealed either lymph node hyperplasia, plasma cell hyperplasia, or lymphomas. An antigen was identified in the lymphomas of mice injected with CD filtrates by indirect immunofluorescence using sera from patients with active CD. Subsequent work suggested that normal and hyperplastic lymph nodes from mice injected with CD filtrates also express an antigen(s) that is recognised by 40–66% of CD sera.^{2–6} Testing with sera from patients with other diseases was usually negative.^{2–5} With regard to antigenic specificity, Das *et al*¹ reported an absence of reactivity of CD sera using *nu/nu* mouse lymph nodes from animals inoculated with control (non-CD)

tissues. In a recent report by Pena *et al*,⁶ however, 18% of the hyperplastic lymph nodes from animals injected with ulcerative colitis (UC) intestinal filtrates were also immunofluorescent positive when tested with CD sera.

The purposes of our study were: (1) to define the histological response in lymph nodes of *nu/nu* mice inoculated with intestinal or mesenteric lymph node tissue filtrates derived from patients with CD, UC, or other intestinal diseases (OID) and (2) to determine the specificity of the 'CD antibody or antibodies' by evaluating under code immunofluorescent reactions between CD sera and lymph nodes from *nu/nu* mice inoculated with CD, UC, or OID tissue filtrates.

Methods

NUDE MOUSE COLONY

An athymic *nu/nu* mouse colony on a *Balb/c* background was established at the University of California, Los Angeles, in 1977 with breeding animals provided by Dr N O Kaplan, University of

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California, San Diego. These animals were housed and maintained as previously described.^{1,2} A total of 175 eight to 12 week old female mice from this colony were entered into the study.

PREPARATION OF TISSUE FILTRATES FROM ATHYMIC MOUSE INOCULATION

Fresh surgically resected mesenteric lymph nodes or intestinal tissues (full thickness) were obtained from patients with CD, UC, or control diseases. This tissue was trimmed of fat, rinsed, and frozen at -70°C . Each tissue was subsequently thawed, minced, suspended in Hank's Balanced Salt Solution (HBSS), and homogenised.¹⁻³⁷ The supernatant fluid was filtered serially through 5.0, 1.2, 0.45, and 0.2 μm Gelman filters (Gelman Sciences, Ann Arbor, MI).^{1,27} The filtrate from each patient's tissue was injected intraperitoneally into six to eight mice, depending on the total amount of protein in each filtrate. Filtrates were not pooled. Each mouse received 0.6–1.4 mg protein from intestinal filtrates or 0.2–0.4 mg protein from lymph node filtrates. The amount of protein was determined by Lowry's method.⁸

The origin of each filtrate was coded and given to the animal technician for injection. Of the 175 mice entered into the study 20 mice served as age matched uninjected controls. One hundred and fifty five athymic mice were injected with the following filtrates: 70 CD tissue filtrates (50 mesenteric lymph nodes, 20 intestine), 37 UC filtrates (27 lymph nodes, 10 intestine), and 48 control filtrates (30 lymph nodes, 11 intestine, seven sham infected).

The diagnoses of CD, UC, and other diseases were determined by review of the patient's history, physical examination, clinical course, laboratory studies and final surgical pathology reports. All patients undergoing surgery for treatment of their Crohn's disease or ulcerative colitis had been on steroids or other immunosuppressant drugs before the surgery. Control tissues were obtained from patients requiring bowel resection for diverticulitis, diverticulosis, adenocarcinoma of the colon (uninvolved tissues), gastrointestinal lymphoma, ischaemic strictures, and normal bowel from patients with traumatic injuries. The variety of intestinal diseases that our filtrates represented allowed us to test for the specificity of the 'CD antibody or antibodies'.

HISTOLOGY OF ATHYMIC MICE TISSUES

Six months after an athymic mouse was injected with a filtrate, the animal was killed and tissues preserved for examination. Of the 175 athymic mice entered into the study, 134 were killed. The tissue from the other 41 mice too was autolysed for analysis. The

gross appearance of the axillary and inguinal lymph nodes, visceral organs, and intestines was recorded. An axillary lymph node and portions of lung, kidney, liver, spleen, and gastrointestinal tract were placed in 10% formalin, sectioned, and stained with haematoxylin and eosin. Adequate histological sections were available on lymph nodes from 97 mice. The lymph nodes were evaluated under code by F Naeim. These findings were corroborated for 33 lymph nodes by Dr A Kadish. Each lymph node was classified as normal or showing lymph node hyperplasia, plasma cell hyperplasia, and/or lymphoma according to previous definitions.¹²

INDIRECT IMMUNOFLOUORESCENT STUDIES OF ATHYMIC *nu/nu* MOUSE LYMPHOID TISSUE

We initially established that we could reproduce the immunofluorescent reaction using a known reactive lymph node from a *nu/nu* mouse injected with a CD filtrate, a positive CD serum, and a negative control serum. These materials were all provided by Dr Das and had been previously tested in his laboratory.

Thereafter, a total of 49 lymph nodes from *nu/nu* mice injected with CD, UC, or control filtrates were evaluated under code by two observers for indirect immunofluorescence. These nodes were selected from the 97 mice on whom we had adequate histology. Without knowledge of histology, one of the authors (MA) randomly selected CD, UC, and OID lymph nodes for immunofluorescence based on the type of filtrate the mouse had received.

SERA USED FOR INDIRECT IMMUNOFLOUORESCENCE

A known positive CD serum and a control negative serum were kindly provided by Dr Das. In addition, we tested three other sera from patients in California and New Mexico with active CD for an immunofluorescent reaction on 31 of the 49 lymph nodes. These patients had all received immunosuppressant drugs. The Crohn's Disease Activity Index (CDAI) in all three of these CD patients was ≥ 200 .⁹

TREATMENT OF SERA

The blood samples were processed and absorbed exactly as previously described.¹² All sera were aliquoted and stored at -70°C . The sera were applied undiluted to the tissue sections.

CONJUGATED ANTISERA

Fluorescein conjugated affinity purified F (ab)₂ fragment of goat antihuman IgG (Cappel 1701–3151) was absorbed in the same manner as the sera, aliquoted and stored at -70°C . An IgG antiserum was chosen because it appeared that the antibody responsible for the positive immunofluorescence in

CD sera was of the IgG type and this is what was used in subsequent studies.^{2 6 10 11} Initially F(ab)₂ fragments of anti-mouse IgG or IgM were used.¹ The sera and FITC-conjugated antiserum were centrifuged on the day of the experiment in a Beckman airfuge at 105 000×g for 20 minutes to remove aggregates.

IMMUNOFLUORESCENCE OF MURINE LYMPH NODES

Axillary lymph nodes from mice inoculated with CD, UC, and control tissue filtrates were selected at random for evaluation of immunofluorescence. Four micron sections of murine lymph nodes frozen in OCT (Tissue Tek II) were cut and processed as previously described.¹² The de complemented and absorbed sera were tested neat and subsequently 15–20 µl FITC-goat antihuman IgG was applied (dilution 1:10 to 1:15) to tissue sections which were incubated at room temperature for 45 minutes in the dark. Slides were washed and dried, and coverslips were mounted with Shandon Immunomount (Shandon-Southern Instruments, Inc, Sewickley, PA). As a control for non-specific immunofluores-

cence an adjacent section of each lymph node was incubated with phosphate buffered saline (PBS) and the conjugated antiserum alone. The antiserum was applied at the same dilution (1:10 to 1:15) used for staining sections with serum and antiserum. Slides were read at ×400 with a Zeiss immunofluorescent microscope. Nodes were interpreted independently under code by two observers and defined as positive if the intensity of immunofluorescence was 2–3+ based on a 0 to 3+ scale.¹ Both observers had to agree on the presence of immunofluorescence or the slide was interpreted as negative.

Results

HISTOLOGY OF *nu/nu* MOUSE LYMPH NODES

Mice were killed six months after injection, except for 26 mice that required necropsy approximately three to five months after injection because of wasting, eye infections, or tumours. Gross inspection of each animal was unremarkable except in two animals. These two mice developed B cell lymphomas involving the liver and/or spleen and lymph

Table 1 Histology of athymic mouse lymph nodes after injection of tissue filtrates

Tissue origin of patients filtrate injected # Patients (n)	Murine nodes examined (n)	Lymph node histology (number of mice)			
		Inadequate tissue	Lymph node hyperplasia	Plasma cell hyperplasia	Lymphoma
Crohn's disease (11)	46	16	7	8	0
Lymph node	32	11	4	3	0
Colon	12	5	3	4	0
Ileum	2	0	0	1	0
Ulcerative colitis (5)	26	9	5	0	1
Lymph node	21	6	3	0	1
Colon	5	3	2	0	0
Colonic adenocarcinoma (1)					
(uninvolved tissue)	16	5	3	1	1*
Lymph node	16	5	3	0	1*
Colon	0	0	0	0	0
Diverticulosis (1)	2	1	0	0	0
Lymph node	0	0	0	0	0
Colon	2	1	0	0	0
Diverticulitis (1)	7	2	3	0	0
Lymph node	7	2	3	0	0
Colon	0	0	0	0	0
Gastrointestinal lymphoma (1)	5	0	0	0	0
Lymph node	0	0	0	0	0
Colon	5	0	0	0	0
Familial polyposis (1)	1	0	0	0	0
Lymph node	1	0	0	0	0
Colon	0	0	0	0	0
Benign stricture (1)	2	0	2	0	0
Lymph node	0	0	0	0	0
Colon	2	0	2	0	0
Sham injected	6	0	1	2†	0
Age-matched controls	18	4	9	0	0
Total	134	37	30	11	2

*Lymphoma in liver and spleen; †One node had both lymph node hyperplasia and plasma cell hyperplasia.

nodes. One occurred in an athymic *nu/nu* mouse that received a UC lymph node filtrate and the other in a mouse injected with the filtrate of an uninvolved mesenteric lymph node from a patient with adenocarcinoma. None of the murine lymph nodes were visibly enlarged. Histologic examination of at least one lymph node from each athymic mouse revealed that 17.1% (30/175) had lymph node hyperplasia and 6.2% (11/175) plasma cell hyperplasia (Table 1). The axillary lymph nodes were examined in 98% of cases. Moreover, almost half of the nodes from the 20 age matched control mice had mild lymph node hyperplasia. Four of these 20 mice had histological evidence of infection. Lymph node hyperplasia occurred without regard to the type of filtrate the mouse had received.

INDIRECT IMMUNOFLUORESCENCE ON *nu/nu* MOUSE LYMPH NODES

Forty five lymph nodes from athymic mice injected with CD, UC, or OID tissue filtrates were evaluated under code for the presence of a transmissible antigen. Lymph nodes from four control mice (two sham injected, two age matched controls) were also tested. The immunofluorescence results and corresponding lymph node histology are listed in Table 2.

The lymph nodes from age matched and sham injected animals were negative when tested with both CD and control sera; in addition, all lymph nodes stained with the control serum were consistently negative. Thirty one to 42 percent of the nodes from mice injected with CD filtrate and tested with CD sera were immunofluorescent positive. Not all of

Table 2 Relationship of indirect immunofluorescence (IFF) results to lymph node histology

Source of tissue filtrate	IFF* results	Total number lymph nodes evaluated	Histology of lymph nodes		
			Lymph node		
			Normal†	hyperplasia	Lymphoma
CD		19			
	Positive	8	1	7	0
	Negative	11	8	3	0
UC		13			
	Positive	4	3	1	0
	Negative	9	7	1	1
OID		13			
	Positive	8	4	4	0
	Negative	5	3	2	0

*Indirect immunofluorescence; †Histologically these lymph nodes were normal or displayed sinus histiocytosis or medullary hyperplasia.

these nodes were positive with all CD sera, even though the Crohn's disease activity index (CDAI) was > 150 in these four patients with CD (Table 3). In addition 17 to 33% of the nodes from mice inoculated with UC filtrates and 14 to 62% of the nodes from OID injected mice were also immunofluorescent positive with CD sera. Immunofluorescent positivity did not correlate with lymph node histology in any of the three inoculation groups (Table 2). The Figure shows positive immunofluorescence using CD serum in a lymph node from a *nu/nu* mouse injected with OID tissue filtrate. The lymph node in this figure was from a *nu/nu* mouse injected with a colonic tissue

Table 3 Results of indirect immunofluorescence (IFF) performed on lymph node sections from athymic mice inoculated with tissue filtrates

Mice inoculated (n)	Source of tissue filtrate Patients (n)	Type of diseased tissue	IFF positive nodes (total number nodes tested)				
			CD serum* 1	CD serum† 2	CD serum‡ 3	CD serum‡ 4	Control serum†
19	CD (5)	Lymph node	5 (13)	2 (9)	2 (3)	—	0 (9)
		Colon	3 (6)	2 (4)	3 (11)	—	0 (5)
		Total	8 (19)	4 (13)	5 (14)	—	0 (14)
		% Positive	42%	31%	36%	—	None
13	UC (2)	Lymph Node	2 (8)	0 (1)	0 (1)	—	0 (1)
		Colon	2 (5)	2 (5)	1 (5)	1 (3)	0 (5)
		Total	4 (13)	2 (6)	1 (6)	1 (3)	0 (6)
		% Positive	31%	33%	17%	33%	None
13	Other intestinal disease (5)	Lymph node	3 (7)	0 (2)	1 (5)	—	0 (1)
		Colon	5 (6)	3 (5)	0 (2)	—	0 (5)
		Total	8 (13)	3 (7)	1 (7)	—	0 (6)
		% Positive	62%	43%	14%	—	None
2	Sham injected		0 (2)	0 (2)	0 (2)	0 (2)	0 (2)
2	Age matched controls		0 (2)	0 (2)	0 (2)	0 (2)	0 (2)
		% Positive	None	None	None	None	None

*Known positive CD serum from Dr Das; †Control negative serum from Dr Das; ‡CD sera from our laboratories (CDAI > 200).

Figure (a) Immunofluorescent staining of a lymph node from a *nu/nu* mouse inoculated with disease control colonic filtrate. Serum used was from a patient with active Crohn's disease. Shown are clusters of medullary cells displaying predominant cytoplasmic fluorescence. Similar positive staining was observed with lymph node sections from Crohn's disease and ulcerative colitis tissue inoculated *nu/nu* mice and Crohn's disease sera.

Figure (b) Negative immunofluorescent staining of same node with a control serum.

filtrate from a patient with a benign stricture. This histologically normal node revealed homogeneous cytoplasmic staining of macrophage-like cells in the medulla.

Forty seven of the 49 nodes tested for immunofluorescence were removed at necropsy six months after the injection of the tissue homogenate or onset of observation (sham inoculated and age-matched controls). One mouse was killed three months after injection of a tissue filtrate from a patient with adenocarcinoma. This athymic *nu/nu* mouse developed lymphoma and the lymph node was immunofluorescent positive. Another animal was killed five months after injection with a UC filtrate because of severe wasting. The node was histologically normal and immunofluorescent negative.

Discussion

Several investigators have proposed that a transmissible antigen is involved in the pathogenesis of CD.¹²⁻¹⁴ The studies by Das *et al.*^{1,2} which have shown immunoreactivity between CD sera and lymph nodes from *nu/nu* athymic mice injected with CD tissue filtrates support this hypothesis. These investigators have suggested that this antigen is specific for CD. Subsequent studies have raised doubt about the specificity of this immunoreactivity between CD sera and lymph nodes from *nu/nu* mice injected with CD tissue filtrates. Pena *et al.*⁶ found that 18% of hyperplastic lymph nodes from *nu/nu* mice injected with UC tissue filtrates were also immunofluorescent positive when tested with CD sera. Likewise, we found that lymph nodes from *nu/nu* mice injected with a variety of control (non-Crohn's disease) tissue filtrates were immunoreactive with CD sera. These results suggest that the immunoreactivity is non-specific. Multiple antigens are probably being transmitted in the tissue filtrates and one or more of these antigens may be CD-specific. We are, however, unable to identify a specific tissue antigen using this experimental mouse model.

The results of our study do not refute the findings of previous investigators that CD sera, and not sera from patients with UC or control diseases, detect an antigen in the lymph nodes of athymic mice injected with CD filtrates. We found that 31-42% of these nodes were immunofluorescent positive, which is similar to the findings of Das *et al.*^{1,2} Moreover, the origin of the tissue injected (colonic or nodal) did not correlate with immunofluorescent positivity.

With respect to the histological response to inoculation with tissue filtrates we found that animals injected with CD tissue filtrates had a similar number of lymph nodes with hyperplastic changes as previous investigators;^{1,2} however, we did note fewer lymphomas. This is probably the result of a shorter time until necropsy¹⁻³ and the use of a 0.20 µm filter to process tissue homogenates. Das *et al.*^{1,2} utilised a 0.45 µm filter² or none at all.¹ Both of these factors could have decreased the overall antigenic stimulation. Cell free tissue filtrates injected by Williams *et al.*,⁷ however, produced five nodes with lymphomas (of 63 injected).

A significant difference, however, was observed in the histology of the lymph nodes from animals injected with control filtrates. In earlier studies^{1,2,7} two of a total of 144 control lymph nodes had either plasma cell or follicular hyperplasia. This contrasts with our 16.6% (13/78) incidence of lymph node hyperplasia in the lymph nodes of our UC injected and OID injected mice. Because the athymic mouse is sensitive to extraneous antigenic stimulation,¹⁵ we

evaluated other factors that might have accounted for this difference. First of all, the mouse colony was not utilised for any other research purposes and has been tested for the presence of pinworms¹⁵ and numerous viruses. Four of the 20 age matched control mice had evidence of wasting (two), pneumonia (one), or nose infection (one). The reactive changes observed in the lymph nodes of these four mice may have been caused by infection but we believe the changes seen in the other control mice were the result of non-specific antigenic stimulation in the environment. We are confident about the interpretation of our histology as the microscopic findings in 33 lymph nodes were corroborated by Dr A Kadish, who carried out the histologic assessment in Das' programme.

We do not think that the immunofluorescent reactivity observed in the control lymph nodes represented a non-specific reaction or autofluorescence as staining with control serum and labelled antiserum was always negative and the cellular location of the staining was specific (Figure). The origin of the type of tissue – that is, whether colonic or from lymph nodes, did not correlate with an immunofluorescent positive reaction. Even though we did not do immunofluorescence on *nu/nu* nodes from mice injected with small bowel tissue filtrates, a previous abstract¹ noted increased immunofluorescent positivity when colonic tissues were utilised. Interestingly, none of the four nodes from athymic mice that were sham injected or age matched controls had any immunofluorescent activity. Even though only four lymph nodes were tested, this observation suggests that the antibody (or antibodies) in CD sera does not cross-react with the antigens derived from the external environment.

In conclusion, our findings support the observations by Das and colleagues in their later work¹ that, regardless of the underlying histology, approximately half of lymph nodes from athymic mice injected with CD filtrates were immunofluorescent positive when tested with CD sera. We do acknowledge that most of the immunofluorescent work by previous investigators has been done on hyperplastic lymph nodes and lymphomas from animals injected with CD filtrates.^{1-3,6,16} We have shown, however, positive immunofluorescence in normal and hyperplastic lymph nodes from mice injected with other types of diseased intestinal or nodal tissues. This implies that the antibody (or antibodies) in CD sera reacts or cross reacts with an antigen common to multiple types of diseased tissues, and that the antigen as tested in the *nu/nu* mouse is not specific for CD tissues.

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