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Immunity to cytopathic agents associated with Crohn's disease: a negative study

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SUMMARY Serum and peripheral blood lymphocytes from 10 patients with Crohn's disease and 10 healthy subjects were examined for immunological reactivity against chick embryo cell cultures displaying cytopathic effects after inoculation with 0·2 μ filtrates prepared from Crohn's disease intestinal tissues. Although the assay systems (indirect immunofluorescence, lymphocyte transformation, and cytotoxicity) yielded positive results using well-characterised cytopathic viruses (mumps, measles), neither Crohn's disease nor healthy subjects showed immune reactivity to the chick embryo cell cultures inoculated with Crohn's disease intestinal tissues in any of the assay systems. These experiments provide evidence against the hypothesis that the *in vitro* cytopathic effect on chick embryo cell cultures produced by Crohn's disease intestinal filtrates are caused by a replicating virus or viruses.

Independent studies from three laboratories have shown that 0·2 μ filtrates of intestinal homogenates from patients with Crohn's disease cause transmissible cytopathic effects when inoculated into a variety of low passage fibroblast-like cell cultures.[1–3] The frequency of this *in vitro* abnormality using Crohn's disease intestine (80–90%) is significantly higher than that seen using disease control intestinal preparations (25%).[3]

Early studies of the properties of this cytopathic effect suggested that it was produced by an RNA-virus.[1–3] More recent work by Phillpotts *et al.*, however, has questioned these findings[4] and subsequently indicated that a soluble toxin(s) may be responsible for the *in vitro* cytopathic effect.[5 6]

In the present studies, we have used an immunological approach in attempting to clarify further the nature of the cytopathic effects induced by Crohn's disease tissue filtrate (CD-TF) in cell culture. We hypothesised that if these effects were viral in nature, viral antigens would be expressed in such cultures and host immune reactivity to these antigens should be demonstrable.

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Methods

PATIENTS

Venous blood was obtained from 10 patients with Crohn's disease (six women, four men) with a mean age 35 years (20–63 years) and 10 healthy adults (five women, five men) with a mean age of 29 years (23–39 years). Among the patients with Crohn's disease six had ileitis, two had ileocolitis, and two had colitis. Eight of the 10 patients had had the disease for more than five years. At the time of blood sampling four patients had active disease, five inactive, and one post-ileal resection two days before. Five patients were taking no drugs at the time of study. The remaining five patients were receiving sulphasalazine (one), sulphasalazine and prednisone (one), or prednisone (three). Serum was separated from an aliquot of the venous sample and stored at −70°C. The remainder of the sample was heparinised and used for lymphoid cell isolation.

CROHN'S DISEASE INTESTINAL FILTRATE PREPARATION (CD-TF)

Bacteria-free and mycoplasma-free 0·2 μ filtrates were prepared from fresh surgical samples of affected intestine.[2 7 8] Tissue filtrates from five different patients with Crohn's disease were selected for the present studies on the basis of their production of virus-like cytopathic effects in chick embryo cells.

TISSUE CULTURE, CD-TF INOCULATION, AND OBSERVATION OF CYTOPATHIC EFFECTS

As chick embryo cells were the most sensitive to the filtrate preparation, most of the immunological experiments were done using these culturtarget cells. Cell culture growth and maintenance media were the same as described by Gitnick *et al.*[2 7] Chick embryo cultures were established from 10-day-old fertile eggs (SPAFAS, Chicago, Ill). Passage two-chick embryo cells at 2×10^4/200 μl growth medium were seeded into the wells of flat bottom microtitre plates (3040 Falcon, Oxnard, Ca). When monolayers were confluent, the medium was removed and the cells were incubated with 50 μl of the filtrate or 50 μl of Hanks balanced salt solution (HBSS) control for one hour at room temperature. Then 150 μl of maintenance medium was added, and the monolayers were incubated at 37°C in humidified 5% CO_2 air. The monolayers were observed for the development of cytopathic effects after the inoculation and scored as follows: − cell appearance similar to control monolayer; + a focal cell detachment over a very small area; ++ definite rounding up cells, with or without cell detachment, over less than half of the well area; +++ cell detachment over more than half of the well area. The same procedure for inoculation and scoring of cytopathic effects was observed in cultures used as positive virus controls. These included chick embryo cells inoculated with mumps virus (Enders strain) at a titre of 10^5–10^6 $TCID_{50}$/ml and Vero cells (American Type Culture Collection, Rockville, Md), inoculated with measles virus (Edmonson strain, American Type Culture Collection) at a titre of 5×10^4 PFU/ml.

ISOLATION OF PERIPHERAL BLOOD LYMPHOID CELLS

Heparinised venous blood was diluted 1:2 with phosphate buffered saline and the lymphoid cells were isolated by Ficoll-Hypaque (Pharmacia, Fine Chemicals, Piscataway, NJ) density gradient centrifugation.[9] Cell viability was over 95% by trypan blue exclusion, and the preparations contained 5–25% monocytes as judged by benzidine staining.[10]

IMMUNOLOGICAL STUDIES

Lymphocyte transformation

Lymphocyte transformation by glutaraldehyde-fixed monolayers Chick embryo monolayers displaying ++−+++ cytopathic effects after inoculation with the filtrate preparation or control monolayers were used as targets for lymphocyte transformation by a modification of the method of McFarland *et al.*[11] Monolayers in flat bottom microtitre plates were fixed with 0·005% glutaraldehyde (Sigma, St. Louis, Mo) for 30 minutes at 4°C and washed three times with phosphate buffered saline. Then $2{\cdot}2\times10^5$ peripheral blood lymphoid cells in 150 μ RPMI $1640_{90}FCS_{10}PS$ were added per well. After four days' incubation, the cells were pulsed for six hours with 0·36 μ Ci [^{3}H]-thymidine (6·7 ci/mmol, New England Nuclear, Boston, Mass) per well, harvested with a multiple automated sample harvester, and counted. Uptakes of [^{3}H]-thymidine were expressed as mean cpm ± one standard error as calculated from quadruplicate samples. Mumps virus (Enders) was used as a positive virus control in this experiment.

Lymphocyte transformation in suspension Crohn's disease intestinal filtrate preparations neat and at dilutions up to 1:8 were used as potential stimulants for 2.2×10^5 peripheral blood lymphoid cells in 150 μl RPMI $1640_{90}FCS_{10}PS$ per well in round bottom type microtitre plates (Nunclon, Nuncatom, Roskilde, Denmark). Additional experiments were carried out using cell culture extracts as potential lymphocyte stimulants as described for human herpes virus infection.[12 13] After the cytopathic effect was manifested, the monolayer was scraped off with a rubber policeman into 0·5 ml Hanks balanced salt solution[12] or 0·043M glycine buffered saline[13] and then subjected to freeze-thawing. Supernatants were collected after centrifugation at 600 *g* for 10 minutes. As a negative control, extracts of uninoculated monolayers were prepared in the same manner. Fifty microlitres of the supernatant were added to $2{\cdot}2\times10^5$ peripheral blood lymphocytes in 150 μl RPMI $1640_{90}FCS_{10}PS$ in round bottom wells. After six days' incubation, the lymphocytes were pulsed with [^{3}H]-thymidine and harvested as described for glutaraldehyde fixed monolayers.

LYMPHOCYTE-MEDIATED CYTOTOXICITY

Chick embryo cell monolayers grown in flat bottom microtitre wells—either uninoculated or inoculated with the Crohn's disease intestinal filtrate preparation—were incubated with medium containing 0·5 μCi of [^{51}Cr] Na_2CrO_4 (50–400 m Ci/mg Cr; Amersham Corp., Arlington Heights, Ill) for six hours, then washed three times with Eagle's minimal essential medium. 1×10^6 peripheral blood lymphocytes in 200 μl $RPMI_{90}FCS_{10}PS$ were added to each well, and, after 16 hours' incubation, a 100 μl aliquot was carefully taken into a tube from each well without disturbing the monolayer in the bottom and replaced with 100 μl of diluted (1:10) Zaponin solution (Coulter Diagnostics Inc., Hialeah, Fla) to lyse the target cells. After an additional 24 hours' incubation, another 100 μl aliquot was taken. These aliquots were counted in a well-type gamma scintillation counter (Packard, Downers Grove, Ill). The percent-

age of ^{51}Cr release was calculated by the following method:

$$\%^{51}Cr \text{ release} = \frac{\text{released } ^{51}Cr \text{ in medium}}{\text{total releasable } ^{51}Cr} \times 100 = \frac{a}{b + \frac{1}{2}a} \times 100$$

where a = cpm obtained from the first aliquot of 100 μl
b = cpm obtained from the second aliquot of 100 μl

This method, in which total releasable ^{51}Cr was obtained for each well tested, permits the calculation of an accurate $\%^{51}Cr$ release. Spontaneous ^{51}Cr release distributed between 25% and 35%. Spontaneous cell mediated cytotoxicity (SCMC) was obtained by the following calculation: SCMC(%)= $\%^{51}Cr$ release obtained from the incubation of peripheral blood lymphocytes and the inoculated or uninoculated monolayer—spontaneous release from the corresponding monolayer.[14] Results were expressed as the mean of the quadruplicates ±one standard error. Measles virus was used as a positive virus control in this experiment.

INDIRECT IMMUNOFLUORESCENCE ANTIBODY TEST

Chick embryo cells, passage 2, were seeded to Lab-tek Tissue culture chamber/slides (No. 4808, Naperville, Ill) and cultured until they were a confluent monolayer. The monolayers were inoculated with 50 μl of the intestinal filtrate preparation, and, after one hour, 250 μl of maintenance medium was added to the chamber. After the appearance of the cytopathic effect, the monolayers were washed with phosphate buffered saline, dried, then fixed with cold acetone for 10 to 15 minutes followed by washing with the saline. Sera from patients with Crohn's disease, healthy subjects, or persons in the acute (CF titre 1:16) or convalescent (CF titre 1:256) stages of measles were tested for antibody by the standard indirect method, using both FITC conjugated goat antihuman immunoglobulins (Antibody Incorporated, Davis, Ca) or FITC conjugated antihuman 1 gM (Cappel Lab, Cochranville, Pa) as the second antibody.

Results

LYMPHOCYTE TRANSFORMATION

Lymphocyte transformation by glutaraldehyde-fixed monolayers (Table 1)

[^{3}H]-thymidine uptake by fixed monolayers alone was negligible (<50 cpm). Peripheral blood lymphoid cells from all nine patients with Crohn's disease and 10 healthy controls showed the expected response to PHA. Those cells from two adults with a previous history of mumps showed substantial transformation in response to the mumps inoculated fixed

Table 1 *Peripheral blood lymphocyte transformation by fixed cell monolayers inoculated with Crohn's disease tissue filtrate (CD-TF)*

Donor	*Disease*	*Unstimulated*	*PHA (2·5 μg/ml)*	*Uninoculated monolayer*	*CD-TF inoculated monolayer*
BT	CD	596±135	6396±267	391±78	367±77
IN	CD	443±90	25080±1575	466±141	387±24
MG	CD	2111±94	33774±717	1390±141	2034±149
MN	CD	1547±40	44375±3767	1482±277	1597±328
AG	CD	763±62	26168±2319	999±51	1235±31
FR	CD	323±51	21342±613	353±9	361±38
CB	CD	604±126	21247±4709	1269±122	1570±194
WM	CD	1024±52	28190±868	954±46	1420±11
JS	CD	1173±89	23230±1709	903±265	1174±90
JE	Control	2304±331	14274±2276	2225±267	2721±322
ST	Control	1185±73	46587±3485	909±105	1286±14
AN	Control	2329±411	16008±743	1939±180	2010±208
MC	Control	1123±97	23526±1062	1161±61	1210±58
LZ	Control	6279±201	23135±1234	5924±632	6374±482
RO	Control	4398±718	47002±1291	9247±829	12642±545
DA	Control	2320±187	54130±5628	3024±400	2932±398
LO	Control	5693±1169	75813±8127	6280±850	8099±1353
LI	Control	1816±158	11639±456	2294±72	2451±328
PA	Control	6465±2278	17840±1708	5411±889	6131±532
					Mumps virus inoculated monolayer
LO	Healthy adults with history of mumps	2932±516	63012±5061	6140±1032	31138±5320
OK		1873±201	16549±1209	1595±181	4050±68

Values indicate mean [^{3}H]-thymidine uptake (CPM)±SEM of quadruplicate samples.

monolayer showing ++ cytopathic effects when compared with uninoculated monolayers. By contrast, the peripheral blood lymphoid cells from the patients with Crohn's disease and from the healthy controls showed no response to the filtrate inoculated fixed monolayers showing equivalent cytopathic effect to that induced by mumps virus. Crohn's disease intestinal filtrate preparations from three different patients were used in these experiments, and each lymphocyte donor was tested against at least two different filtrate inoculated monolayers. Peripheral blood lymphocytes from two patients with Crohn's disease and four controls were harvested on the fourth, fifth, and sixth days of incubation with the cell monolayers, and again no response was observed.

Lymphocyte transformation by Crohn's disease tissue filtrate and cell culture extracts

This filtrate when used undiluted significantly inhibited [^{3}H]-thymidine uptake by the peripheral blood lymphocytes from four patients with Crohn's disease and four healthy subjects (Table 2). In four subjects tested, the inhibitory effect was substantially decreased by serial dilution of the filtrate (Table 2). In three subjects (one Crohn's disease, two controls), all five filtrates were tested. Spontaneous [^{3}H]-thymidine uptake in these three individuals was uniformly inhibited by all five filtrates. This inhibitory effect of the Crohn's disease tissue filtrate was not eliminated by heat treatment (65°C, 20 minutes) or by ultraviolet irradiation. The responses of four patients with Crohn's disease and one healthy control were not altered by incubation with GBS cell extracts derived from two filtrate inoculated cultures or to the same cell extracts without GBS treatment (Table 3).

Lymphocyte-mediated cytotoxicity

Peripheral blood lymphoid cells from 10 patients with Crohn's disease and nine healthy subjects were each tested against at least two monolayers inoculated with Crohn's disease tissue filtrates. The lymphoid cells from most donors showed some spontaneous cell mediated cytotoxicity over that due to spontaneous release. There was, however, no difference in this cytotoxicity in inoculated compared with uninoculated monolayers (Table 4). By contrast, using the same assay system, lymphoid cells from healthy subjects with a history of measles showed higher cytotoxicity against vero cells infected with measles virus than against uninfected vero cells (Table 4).

Indirect immunofluorescence antibody test

Ten sera from patients with Crohn's disease and 10 sera from healthy subjects were tested at a 1:10 dilution against monolayers of chick embryo cells inoculated with two different Crohn's disease tissue filtrates and showing + − + + + cytopathic effect. In both uninoculated and Crohn's disease tissue filtrate

Table 2 *Peripheral blood lymphocyte transformation in response to Crohn's disease tissue filtrates (CD-TF)*

Donor	*Disease*	*No. of CD-TF tested*	*Unstimulated*	*Stimulated with CD-TF*			
				Neat	*1:2*	*1:4*	*1:8*
CB	CD	2	152±21	62±6	NT	NT	NT
IN	CD	2	1176±180	287±118	NT	NT	NT
BT	CD	1	283±143	79±18	NT	NT	NT
GM	CD	5	286±79	39±9	104±7	160±22	299±67
PA	Control	5	420±51	41±19	67±8	155±30	287±82
MC	Control	5	343±53	47±15	91±13	153±14	273±46
JE	Control	2	1030±219	488±72	NT	584±301	730±194
ST	Control	2	2040±264	942±192	NT	NT	NT

NT: not tested.
Values indicate mean [^{3}H]-thymidine uptake (CPM)±SEM of quadruplicate samples.

Table 3 *Peripheral blood lymphocyte transformation in response to GBS and non-GBS extracts of cell cultures inoculated with Crohn's disease tissue filtrates (CD-TF)*

Donor	*Disease*	*Unstimulated*	*Extract*	*Tissue culture extract stimulated*		
				Uninoculated	*Inoculated with CD-TF[1]*	*Inoculated with CD-TF[2]*
MN	CD	448±122	GBS	332±71	326±29	356±32
MG	CD	487±79	GBS	445±41	379±10	417±91
MC	Control	1092±41	GBS	1663±244	2096±336	2210±86
AG	CD	1223±204	Non-GBS	1190±370	1228±358	1471±309
FR	CD	2003±217	Non-GBS	2542±241	2162±309	2183±496

Values indicate mean [^{3}H]-thymidine uptake (CPM)±SEM of quadruplicate samples.

Table 4 *Peripheral blood lymphocyte (PBL) cytotoxicity (SCMC) for ^{51}Cr-labelled chick embryo cell monolayers uninoculated or inoculated with Crohn's disease tissue filtrates (CD-TF)*

Donor	*Disease*	*Uninoculated*	*Inoculated (CD-TF1)*	*Inoculated (CD-TF2)*
GM	CD	4·2	2·2	8·3
WM	CD	16·2	19·5	15·5
IN	CD	39·6	28·9	29·3
BT	CD	5·9	5·9	2·6
MG	CD	2·5	0	0
MN	CD	2·1	1·7	NT
AG	CD	1·4	2·7	1·9
FR	CD	0·6	0·1	0·9
CB	CD	0	1·0	0·8
JS	CD	0·2	0·7	0
MC	Control	6·5	0	3·7
PA	Control	4·1	3·0	6·5
ST	Control	2·6	0	0
JE	Control	15·0	8·3	8·4
AN	Control	7·3	5·8	5·1
LZ	Control	0	2·0	0
RO	Control	0	0	0·7
DA	Control	6·4	4·3	1·6
LO	Control	8·7	6·3	6·8
		Uninoculated vero cells	*Measles virus inoculated vero cells*	
MS	Healthy adults with history of measles	4·3	15·7	
MC		5·7	17·6	

% SCMC = % ^{51}Cr release from monolayer incubated with PBL − spontaneous. ^{51}Cr release from monolayer alone.

inoculated monolayers all sera caused faint, diffuse cytoplasmic staining. Neither an increase in the intensity of the fluorescent staining nor an increase in the number of stained cells was seen in monolayers inoculated with the filtrate. Serial serum dilutions (1:5–1:60), the use of goat anti-IgM as second antibody or the use of another cell culture system susceptible to the Crohn's disease filtrate (WI-38) did not result in significant fluorescent reactions. By contrast, in the virus control experiment, sera (1:10–1:60 dilutions) from two patients with measles in either the acute or convalescent stage showed bright staining against measles infected vero cells and no staining against uninfected control monolayers.

Discussion

Previous studies of the *in vitro* cytopathic effect of Crohn's disease intestinal filtrates have suggested that it is caused by an RNA-virus associated with the diseased bowel of such patients.[1 2 3] Thus, the cytopathic effect is transmissible in cell culture, unaffected by heat, ether, acid, or IUdr and virus-like particles have been described in cultures undergoing this effect.[1 2 3] If this effect is caused by a cytopathic virus, then, by analogy with other known virus systems, host immunity to the agent should be readily demonstrable. Thus in the present studies we have used several established techniques in attempting to show cellular and humoral immunity to putative virus(es) present in cell cultures undergoing cytopathic effect after inoculation with Crohn's disease intestinal filtrates.

Glutaraldehyde-fixed cell monolayers displaying cytotoxicity after inoculation with mumps, vaccinia, and measles viruses have been shown to elicit lymphocyte transformation, using peripheral blood lymphoid cells from subjects previously sensitised to these viruses.[11] In the present study we have confirmed this finding using mumps virus infected fixed cell monolayers. Using identical cell monolayers undergoing cytopathic effect in response to inoculation with Crohn's disease intestinal filtrates, however, no transformation of lymphoid cells from patients with Crohn's disease or healthy controls was observed (Table 1).

We also attempted to elicit lymphocyte transformation in suspension using the Crohn's disease intestinal filtrate and cell extracts from cultures undergoing cytopathic effect after inoculation with this filtrate. The use of these extracts was predicted on evidence indicating that cell membrane-associated viral antigens are of key importance in the production of *in vitro* lymphocyte transformation.[14 15] Such extracts have been successfully used to demonstrate lymphocyte transformation in human herpes virus infection.[12 13] In the present study, the undiluted filtrate actually caused consistent inhibi-

tion of lymphocyte transformation, and the filtrate inoculated cell extracts yielded no response either stimulatory or inhibitory (Tables 2, 3).

The *in vivo* significance of *in vitro* lymphocyte-mediated cytotoxicity against virus infected target cells remains uncertain. Nevertheless, this *in vitro* assay has been shown to reflect lymphocyte (T-cell, N-K cell, or both) reactivity in several established viral infections in man.[14 16 17] Using peripheral blood lymphoid cells from subjects exposed to measles, we have confirmed the presence of enhanced cytotoxicity against cell cultures infected with measles virus. By contrast, using these cells from control subjects or patients with Crohn's disease in a similar assay system incorporating cell cultures inoculated with Crohn's disease intestinal filtrates, no enhanced cytotoxicity was demonstrable (Table 4). As these experiments used xenogeneic target cells, the major function measured by these assays was probably that of N-K activity, rather than T-cell cytotoxicity.

Our previous attempts to detect antibody to the putative viral agents in Crohn's disease necessitated serum neutralisation of the cytopathic effect.[3] These studies showed inhibition only at low serum dilutions, and no differences were observed between sera from patients with Crohn's disease and normal sera.[3] The present results using immunofluorescence also indicate the absence of apparent antibody activity in sera from patients with Crohn's disease or normal sera towards antigens expressed in cell cultures undergoing the cytopathic effect after inoculation with Crohn's disease intestinal filtrates. These findings are in agreement with those recently reported by Phillpotts *et al.*[5 6]

Thus, using established methods for detecting cellular and humoral immunity to known cytopathic viruses in culture, we have been able to show such reactivity to the proposed agents associated with Crohn's disease previously implicated in causing *in vitro* cytopathic effects. The number of donors studied (10 with Crohn's disease, 10 controls) was not large, but the consistency of the findings makes subject selection an unlikely explanation for the negative results. Could the findings be attributable to a more generalised depression of cellular immune function in Crohn's disease? Previous studies of cellular immunity in Crohn's disease have been in sharp conflict with regard to the presence or absence of T-cell deficiency[18] Moreover, in the present study there was no significant difference in PHA responses between control or Crohn's disease PBL. Recently, N-K activity has been reported to be reduced in Crohn's disease,[19] and possibly our negative findings using cytotoxicity could reflect deficient N-K function. This possibility would not, however, explain the negative results obtained using transformation assays.

In conclusion, we suggest that the results of the present study raise considerable doubt about the proposed viral causation of the *in vitro* cytopathic effect induced by Crohn's disease tissue filtrates. Very recent studies by Phillpotts *et al.*[5 6] and McLaren *et al.*[20] also provide evidence against this proposal. These workers have shown that many of the properties of the cytotoxicity induced by these filtrates are more consistent with a tissue associated toxic effect. Indeed, the inhibitory effect of Crohn's disease tissue filtrates on lymphocyte function observed in the present study may also represent such a toxic effect.

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