THE CELLULAR MEDIATED IMMUNITY TO LYMPHOGRANULOMA VENEREUM AGENT

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ABSTRACT

The chlamydia are well-known and widely spread throughout the world. However, little is known about the immune responses to these agents or their underlying mechanisms. The objective of this research was to examine the mechanisms involved in the dermal reaction to chlamydial agents.

Using in vitro techniques for the investigation of the cellular response such as the Macrophage Inhibition Test and the Macrophage Spreading Inhibition Test, it was discovered that lymphogranuloma venereum infection produced a cellular immune response that manifested itself in a dermal reactivity.

Examination of the LGV agent by immunochemical methods showed that several components of the LGV agent are capable of producing a cellular immune response; these components were studied for protein nitrogen and carbohydrate content.

It was concluded from this work that some chlamydia have a cellular immune reaction as part of their total immune response and that more than one structural component of the agent is acting to initiate that response. It was also demonstrated that cytophilic antibodies do not seem to play a role in the cellular mediated reaction.

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INTRODUCTION

The chlamydia are intracellular parasites that belong to the order Rickettsiales. They have a distinctive reproductive cycle that includes two forms, the elementary body (EB) which is the infectious form, and the initial body (IB) which is the intermediary form associated with replication (Bergey's Manual; Meyer, 1965).

Among other agents, the group includes the agents of psittacosis, lymphogranuloma venereum, meningopneumonitis, ornithosis, mouse pneumonitis, cat pneumonitis and trachoma. The agents in this group are closely related in some ways and share common heat-stable antigens. Due to the presence of the common antigens, the agents are capable of cross-reacting to various degrees in a variety of tests, such as complement fixation, haemagglutination, toxin neutralization, infectivity neutralization, immunofluorescence and skin testing (Kilham, 1948; Meyer, 1965).

In chlamydial infections, the long-term molecular interaction between the host and the agent is not clear. Latency, or a chronic subclinical infection, where the organism remains inside the cell for long periods of time, has been suggested as a cause of the high and rather long-lasting antibody levels to chlamydia among the Eskimos in Northern Canada (Wyman et al., 1970). Meyer (1965) suggested that this latency is

is due to a complexity of factors, among them is the lack of nutritional requirements of the agent. This state of chronic, subclinical infection, or latency, has also been suggested as the cause of the dermal reaction that is associated with chlamydial group infections (Bietti et al., 1967; Meyer, 1968). It seems that many chlamydia tend to persist in the tissues of the host in a latent state and to elicit in man and animals a state of cutaneous hypersensitivity revealed by a delayed type response when a suitable antigen is injected intradermally (Bietti et al., 1967).

This skin reaction has been known for many years and has been studied in varying detail. Experiments with five of the agents: LGV, psittacosis, cat pneumonitis, meningopneumonitis and mouse pneumonitis, showed a high degree of cross-reactivity in both complement fixation (CF) tests and in skin tests (Kilham, 1948). The degree of reactivity found in the two tests, however, depended on the route of inoculation: either intracerebral or intratracheal. For psittacosis, either route produced a 1:16 to 1:64 titre in the CF test with antisera against the other four agents, this was not so for the other agents. With the skin tests, the intratracheal route showed higher degrees of dermal reactions for all five agents, as compared to the intracerebral route. Kilham concluded from these results that a better correlation for cross-reactivity was obtained with the complement fixation test, rather than the skin test, irrespective of the

route of inoculation. The differences in correlation may have been due to the relative sensitivity of the two tests, complement fixation being more sensitive. However, Barwell (1952) found that the antigens reacting in the two tests were different. It was shown that the complement fixing activity of the heat-stable antigen is destroyed by periodate treatment without any loss in the skin reactivity. Benedict et al. (1958) showed that individuals infected with psittacosis showed a marked increase in the passive haemagglutination titre after skin testing. It was thought that the skin initiated an anamnestic response, increasing the haemagglutination reaction. They were also able to show that the antibodies for the complement fixation and haemagglutination reactions were different. It seems that the antigens for the three reactions are not related and have a varied effect on the immune response of the host.

Results using both skin tests and complement fixation tests with trachoma antigens also showed cross-reactivity in both tests (Bietti et al., 1967). It was found that positive skin tests were obtained in persons who did not have a clinical infection, or who had a low serum antibody level. Positive dermal reactions were also obtained from persons living in an area where the disease was endemic. The same authors were able to transfer the hypersensitive state to non-reactive individuals by the intradermal injection of leucocytes obtained from positive donors.

There have been few attempts to study this hypersensitive state and its relationship to disease. In certain diseases such as tuberculosis or lymphogranuloma venereum, the hypersensitive reaction has a damaging effect on the tissues and constitutes a main factor in the pathogenesis of both diseases (Boyd, 1956). This is indicated by the nature of the lesions in the lung tissue of persons with tuberculosis. The role of the hypersensitive state in the pathogenesis of psittacosis has not been studied. Meyer (1965) has described the pathology of psittacosis infections as being similar to those of tuberculosis. The histopathological picture of fully developed lesions in psittacosis and ornithosis has been described as an accumulation of lymphocytes and macrophages in the alveolar-spaces with a scarcity of polymorphonuclear leucocytes in the alveolar exudate (Meyer, 1965). In lymphogranuloma venereum, the inflammatory process consists of an outpouring of plasma cells, few neutrophils and eosinophils and an increase of macrophages with transformation. Epitheliod transformation of the macrophages gives rise to peculiar tubercle-like nodules which undergo necrosis.

The conclusions of the previously mentioned researchers indicates that chlamydial infections may cause a cellular mediated reaction of the delayed hypersensitivity type in the host. However, whether it is a cellular immunity mediated by lymphocytes and macrophages as in tuberculosis or an antibody mediated reaction, has not been studied. This was

the problem to be investigated in this project.

First, it is necessary to state the guide-lines to be followed in the examination of this problem. Sell and Asofsky (1968) characterized delayed hypersensitivity by the following criteria:

1) delayed hypersensitivity can be transferred only by specifically sensitized cells, not with serum or a subcellular fraction.

2) the time for the skin reaction is more prolonged than in other allergic states, and may last for days or weeks.

3) the histological picture of the reaction site differs in different allergic responses. In anaphylaxis, it is mainly edemous with little or no cellular infiltrate. In the Arthus reaction, there is an influx of polymonphonuclear leucocytes and fibrinoid necrosis occurs. In delayed sensitivity there is an infiltration of cells, mostly monocytes, at the site of the antigen, with subsequent tissue damage (Bloom and Chase, 1967).

Until recently, the skin reaction and the histopathological picture of the site of the reaction were the only methods available to study the delayed response. Using a method devised by George and Vaughan (1962), David et al., (1964) were able to use an in vitro technique to study the cellular mediated immunity to tuberculosis. The test is based on the concept that specifically sensitized macrophages are inhibited from their normal migration out of a capillary tube in the presence of specific antigen (1964a).

These workers found that the macrophages obtained from animals sensitized with old tuberculin were inhibited from migration when the sensitizing antigen (PPD), was incorporated into the culture medium. Many aspects of cellular mediated immunity have been described through this technique. First, it was found that the reaction was specific for the sensitizing antigen (David et al., 1964a). Second, cells which produce precipitating antibodies were not inhibited by the antigen (David et al., 1964b). Third, it was shown that DNP and an unrelated protein carrier, or an unconjugated carrier had no reactivity; only the specific hapten and carrier would give a reaction and inhibit migration of macrophages (David et al., 1964c). In other immunological reactions the hapten is important, in a cell mediated reaction it would seem that the carrier or the carrier and hapten conjugate is important. Fourth, only a few specifically sensitized lymphocytes are required. It was found that in a population of one hundred cells, only two of these need to be lymphocytes, the rest are macrophages (David et al., 1964c).

Fauve and Dekaris (1969) modified the test to a more convenient slide test. This technique was based on the theory of macrophage inhibition (MI). The Macrophage Spreading Inhibition test (MSI) is a direct quantitation of the numbers of macrophages spreading on a glass surface. Hemocytometers were used for this purpose. Fauve and Dekaris (1969) were able to show that the results obtained by using this

technique were reproducible under the conditions applied. The technique has the advantages of less time and material used since the MSI may be completed within two hours while the MI requires two days for completion.

Using the MI and the MSI, David et al. (1968) and Fauve and Dekaris (1970) respectively have studied the relationship between the in vitro tests and the in vivo skin reactions by comparing the results obtained in the MI and MSI with the degree of dermal reactivity. A high degree of cellular sensitivity as shown by the MI test compared with a highly reactive skin test in an individual and vice versa (David et al., 1968). Using guinea pigs, Fauve and Dekaris (1970) were able to show the same results when the MSI was used.

The mechanism controlling the MI is controversial. A factor, called the macrophage inhibiting factor (MIF) was found to be in the medium of sensitized cells incubated in vitro with specific antigen (Bloom and Bennet, 1966; David et al., 1966). The factor has the ability to inhibit the migration of normal non-sensitized cells. It is produced specifically by the sensitized cells in the presence of the specific antigen. The MIF is non-dialysable, has a molecular weight of approximately 150,000 and is active after incubation at 56°C for thirty minutes (Bloom and Bennet, 1966, 1968; David et al., 1966).

Recently a leukotactic factor (LF) was found closely associated with the MIF. It is produced along with the MIF in vitro in the presence of the

sensitizing antigen. The LF was isolated by gel chromatography using Sephadex G-100 (Ward and Remold, 1969). It was found to be immunologically specific in action and is produced under similar conditions as those of the MIF.

The biological activities of the two factors, MIF and LF, have been studied. The MIF is known to produce a delayed-type skin reaction when injected intradermally. It also stimulates normal lymphocytes to divide and transform and can be shown to contain newly formed antibody (Bloom and Bennet, 1968). However, the LF had not been identified at the time of that study and it is possible that the LF was present with the MIF as a contaminant. It is thought that the LF may be responsible for the large number of non-sensitized mononuclear cells seen at the reaction site. It was postulated that the LF attracts the extra cells to the site while the MIF stops the cells from migrating away (Ward and Remold, 1969). Heise and Weiser (1969) studied a cytotoxin produced by tuberculin sensitized lymphocytes and its effect on mouse L-cells. Pincus (1967) had reported that both normal macrophages and those from tuberculin sensitized guinea pigs would release a cytotoxin when cultured with PPD. Heise and Weiser (1969) found that only sensitized lymphocytes were specifically stimulated by the antigen to release the cytotoxin. The reasons behind this phenomenon are not clear. The authors related the phenomenon to the tissue damage that occurs in delayed reactions.

While no circulating antibody has been shown to have a role in cellular mediated immunity (David et al., 1964, 1966), cytophilic antibody has not been entirely ruled out. David (1964) was not able to detect the presence of antibody in his MI system. However, Heise et al. (1968) found that the supernatant from sensitized cells incubated at 56°C for thirty minutes would inhibit the migration of normal macrophages. Similarily, if trypsin were applied to the sensitized cells, their migration would no longer be inhibited by the presence of specific antigen. Trypsin was also found to destroy the inhibitory effect of the heat-treated supernatant mentioned previously.

While all these aspects have greatly increased our understanding of the nature of cellular mediated immunity, one factor is yet to be determined; that is, the benefit derived by the host from such an immune mechanism. The purpose of this type of immunity is, as yet, unknown (Boyd, 1956).

MATERIALS AND METHODS

A. Agent and Antigens

1) Agent

Lymphogranuloma venereum antigen was obtained from Markham Laboratories (Chicago, Ill.). The agent was grown in tissue culture, inactivated, and purified. The material was standardized for nitrogen content (Lowery, 1951) and complement fixation titre (Meyer, 1969) in our laboratory.

2) Skin test antigen

A skin test antigen of Lymphogranuloma venereum was obtained from Lederle (Cyanimid of Canada, Winnipeg, Manitoba). The skin test set contained 1.0 ml. of test antigen and 0.1 ml. of control test saline. The agent was cultivated in chick embryo, inactivated and suspended in physiological saline containing 0.3% phenol and 0.1% formalin. The content of the sonicated agent was 27.8 mg. per milliter.

B. Animals

1) Guinea pigs

The Hartley-strain and random bred female guinea pigs (Canadian Breeding Farm) were used for the Macrophage Inhibition test (MI) and for skin testing.

2) Mice

Female mice of the CF_1 strain (home bred) were used in tests for the macrophage spreading inhibition (MSI) test.

C. Sensitization of Animals

1) Guinea pigs

Twelve guinea pigs were sensitized with a single dose of 0.05 ml. Lymphogranuloma venereum (Markham) in 0.05 ml. of Freund's complete adjuvant through inoculation in the foot-pad.

2) Mice

Female CF_1 mice were used for testing with whole Lymphogranuloma venereum and for the fractions of the sonicated agent obtained from the LKB electrofocusing columns. A total of 104 mice were used for immunization by fractions of the agent obtained from the columns, and 6 mice were used for the whole agent. For sensitization, a single injection of 0.05 ml. of LGV (Markham) in Freund's complete adjuvant was injected in the hind foot-pads.

D. Skin Test

At 2, 3 and 4 weeks after inoculation of the animals, 2 guinea pigs and a control animal were skin tested. An area, approximately 3-4 inches square on the back of the animals was shaved, and 0.1 ml. of the skin test antigen was injected in 3 different areas. A fourth

site was used for the control injection. Results were read after 24, 48 and 72 hours after the intradermal injection. The dermal reaction was determined by measuring the two axis of the area of erythema and induration to obtain an approximation of the area of the reaction.

E. Macrophage Inhibition (MI) Test

Three weeks after sensitization and three days before performing the MI test, 30 ml. of sterile Bayol oil (Esso) was injected intraperitoneally into the guinea pigs (Uhr and Pappenheimer, 1956). After three days the animal was sacrificed by bleeding it out by cardiac puncture. Chloroform was not used as it tends to have a detrimental effect on the metabolism of the macrophages. At this time 100-150 ml. of sterile Hank's balanced salt solution was injected into the abdomenal cavity. The abdomen was then massaged to remove the oil from the walls of the cavity. A perforated trocar (17 French, Stevens and Co.) was used to drain the cavity into a sterile 250 ml. separatory funnel. The funnel was shaken to allow the oil to separate and placed in the cold for 30 minutes, or until the oil had separated. The cells were collected in 50 ml. round-bottomed centrifuge tubes and centrifuged at 4°C for 10 minutes in a Servall Superspeed RC-2 refrigerated centrifuge at 1200 rpm. The

cells were washed twice with Hank's BSS and centrifuged for 5 minutes at 1000 rpm at 4°C. The cells were resuspended in 0.9 ml. Eagle's minimal essential medium containing 15% normal guinea pig serum, to 0.1 ml. packed cells. The cell suspension was kept in an ice-bath to minimize the cellular metabolism. Capillary tubes (7.5 mm. x 1.5 mm., non-heparinized) were filled with the cell suspension and sealed at one end by heating. The capillary tubes were spun at 900 rpm at room temperature for 5 minutes in an IEC International centrifuge (Universal Model UV). The capillary tubes were then cut slightly below the packed cell-liquid interface and fastened inside an incubating chamber (1.9 cm. in diameter and 0.6 cm. deep) with silicone grease. Two capillaries were placed in each chamber. The chamber was then covered with a sterile cover glass and sealed with paraffin wax. The chambers were filled with approximately 2.0 ml. of Eagle's MEM and 0.1 ml. of antigen containing 50 ug protein nitrogen (skin test antigen-Lederle) was added to the appropriate chambers. The chambers were incubated at 37°C for 24 hours. The area was determined by using a grid-ocular on a low power microscope (Olympus tissue-culture microscope, model 200748). The MIT indices were calculated according to the formula (David et al., 1964).

$$\text{MIT} = \frac{\text{area of migration with Ag}}{\text{area of migration without Ag}} \times 100$$

For each individual test, the final MIT index was determined by the average area of 4 capillaries.

F. Macrophage Spreading Inhibition (MSI) Test

Three weeks after sensitization of the mice, the peritoneal cells were collected. The animals were sacrificed by ether inhalation and 5.0 ml. of medium 199, containing 20 mg. bovine serum albumin (BSA) and 5 units of heparin per milliter, was injected intraperitoneally. The abdomenal cavity was massaged gently and the exudate was withdrawn with a 23 gauge needle and centrifuged at room temperature for 5 minutes at 1000 rpm on an IEC centrifuge. The cells were then resuspended in 0.6 ml. of medium 199 and divided into two portions in sterile test tubes. To each, 0.1 ml. of test antigen containing 50 ug. of protein nitrogen was added. The tubes were incubated in a water-bath at 37°C for 30 minutes. The cells were then placed on a hemocytometer and incubated in a moist chamber at 37°C for 30 minutes. The slides were then examined under phase contrast microscopy (Zeiss Photomicroscope, lens 16/0.40). The results were determined by taking the average percentage of spreading cells incubated with and without antigen

(Fauve and Dekaris, 1968).

$$MSI = \frac{\%\text{ cells spreading with Ag}}{\%\text{ cells spreading without Ag}} \times 100$$

The MSI index for each test was obtained by calculating the average indices of five sets of experiments.

G. Electrofocusing

1) Sonication

Before applying the inactivated agent to the column, the agent (skin test antigen-Lederle) was sonicated for 30 minutes at 300 Kc in a Biosonik III (Bronwill Scientific, Rochester, N. Y.) at 4°C. The purpose was to rupture the cell, thus exposing as many antigenic components as possible. The sonicated material was checked by electron microscopy for the presence of intact agents.

2) Electrofocusing procedure

Electrofocusing was carried out according to the instructions in the LKB 8100 Ampholine Instruction Manual on both the LKB 8101 and 8102 columns (Hagbend, 1967). A three ml. sample of sonicated agent was applied to the 8101 column or a five ml. sample applied to the 8102 column. LKB Ampholine Carrier Ampholytes of pH range 3-10 were used. The columns were run at 10°C (cold-water circulating in the outer jacket of the column throughout the run) with wattage maintained between 2-4 watts. The runs continued for 48 hours. The

column was eluted using a polystatic pump (Buchler Instruments, 115V) at a flow rate of 1. 2 ml. per minute. Fractions were collected in 3 ml. fractions for the 8101 column and 7 ml. for the 8102 column. The pH was read immediately. The fractions were dialysed against tap water for 3 days to remove ampholytes and sucrose. Optical density was read at 280 mu on a Unicam SP500 Series 2 Spectrophotometer, and graphed. Fractions were pooled according to O. D. peaks at 280 mu and concentrated either by air drying or by dialysis against 20% PVP. The peaks were then tested for chemical nature and their MSI activities.

H. Disc-Gel Electrophoresis

Prior to use, the electrophoresis tubes were acid cleaned, rinsed with tap and distilled water and finally rinsed with dilute Kodak Photo-Flo 200 and allowed to dry. One end of each tube was sealed and the tube placed in the tube holder assembly. The tubes were adjusted so that the bottom of the tubes were level with each other and the assembly was leveled.

Each tube was filled with freshly prepared lower gel solution to an approximate height of 40 mm. using a Pasteur pipette. Distilled water was then carefully layered on top of the gel in order to polymerize the gel. The gel solution was allowed to polymerize for

approximately 30 minutes. The water was then carefully removed.

Approximately 0.15 ml. of freshly prepared upper gel solution was then added to each tube. Distilled water was carefully layered on top. A fluorescent light source was placed three inches behind the assembly and the upper gel solution was allowed to photopolymerize for 15 minutes. The water was then removed.

After polymerization the sealed ends were removed and the tubes placed in the electrophoresis chamber. The tubes were adjusted so that they were immersed a distance of 2 cm. each into the lower buffer chamber. Sufficient upper buffer was added to cover the upper electrode. The lower buffer chamber was three-quarters filled with lower buffer.

0.075 ml. of the sample solution was carefully layered on top of the upper gel. The density of the sample had previously been increased with 5-10% sucrose. A buffer front was marked by the addition of 1 ml. of 0.001% bromophenol blue to the upper buffer.

A current of 1.25 ma. per tube was applied until the samples had entered the stacking gel. The current was then increased to 2.5 ma. for the remainder of the run. The current was shut off when the buffer front was approximately 2 mm. from the bottom of the tubes. The gel columns were then removed from the tubes by a 22 guage

needle in a distilled water bath.

The gel columns were stained with 0.1% Naphthol blue black in 7% acetic acid for 30 minutes. The gels were placed in destaining tubes and placed in the electrophoresis chamber. The upper and lower buffer chambers were filled with 7% acetic acid. A current of 5 ma. per tube was applied until all the stain was removed - approximately 1 hour.

The columns were compared for the presence of identical bands of protein.

I. Cytophilic Antibody

The MSI was carried out as previously described, with a slight variation. Sensitized peritoneal cells were incubated at 56°C for 30 minutes. The cells were then centrifuged at room temperature for five minutes at 900 rpm. The supernatant was taken off and the cells discarded. This supernatant was then incubated with normal peritoneal cells from mice, at 37°C for 30 minutes. The MSI was then carried out as previously described, using the fractions from the smaller electrofocusing column as antigens.

J. Chemical Analysis

a) Protein Determination

Protein nitrogen was determined by the Lowery Method (Lowery et al., 1951).

b) Non-reducing sugars

Non-reducing sugar was determined by the Fairbairn modification (1953) of the Dreywood anthrone test (1946).

RESULTS

I. Skin Test

It can be seen from Table I and figure 1, that the animals had already obtained a level of sensitization to the LGV agent 2 weeks after injection with the agent. However, the degree of dermal reactivity increased with time as shown by the results obtained after 4 weeks. Each skin test was measured after 24, 48 and 72 hours. No distinct pattern can be seen from following the size of the skin reaction. The animals tested after 2 weeks sensitization started with a reaction 0.60 mm.^{2} after 24 hours and 0.69 mm.^{2} after 72 hours. Similarily, after 4 weeks of sensitization the reaction after 24 hours was 0.97 mm.^{2} and 0.53 mm.^{2} after 72 hours. This discrepancy in reaction size could be due to the degree of sensitivity obtained by each animal. A reading of the skin test after 48 hours would help to minimize this effect, from a diagnostic point view. In all cases the control injections of saline in sensitized animals showed negative reactions.

II. Macrophage Migration Inhibition (MI) Test

Results shown in Table II show the MI indices ranging from 30.7 to 73.5 per cent over a period of seven weeks. As the period of sensitization increased from two to four weeks the MI indices

decreased to 30.7 per cent from 61.8 per cent. This meant that while the MI indices decreased, this was in reality, an increase in cellular sensitivity, as the lower the MI index, the higher the degree of cellular reactivity. After the four week period the MI index increased such that at seven weeks the MI index was 73.5 per cent. Thus an optimum period of 4-5 weeks is required to obtain the best results for in vitro testing and shows the optimum cellular immune response. This optimum time of cellular activity in vitro coincides with the in vivo reaction obtained with the skin tests, which showed a better reaction after a 4 week period of sensitization, rather than after a two week period.

III. Macrophage Spreading Inhibition (MSI) Test

Table III shows the results of the MI tests. These tests were done to compare the MI and MSI indices. Figure 2 shows a comparison between the two tests with respect to time and biological activity of cells. The two tests compared favourably considering the different methods and animals used, i.e., the MI used guinea pigs, and the MSI used mice. Table III shows that a similar pattern of sensitization was obtained with the MSI as with the MI in Table II. With the MSI, using mice, the optimum time of sensitization was obtained after two weeks, with a MSI index of 50.0 per

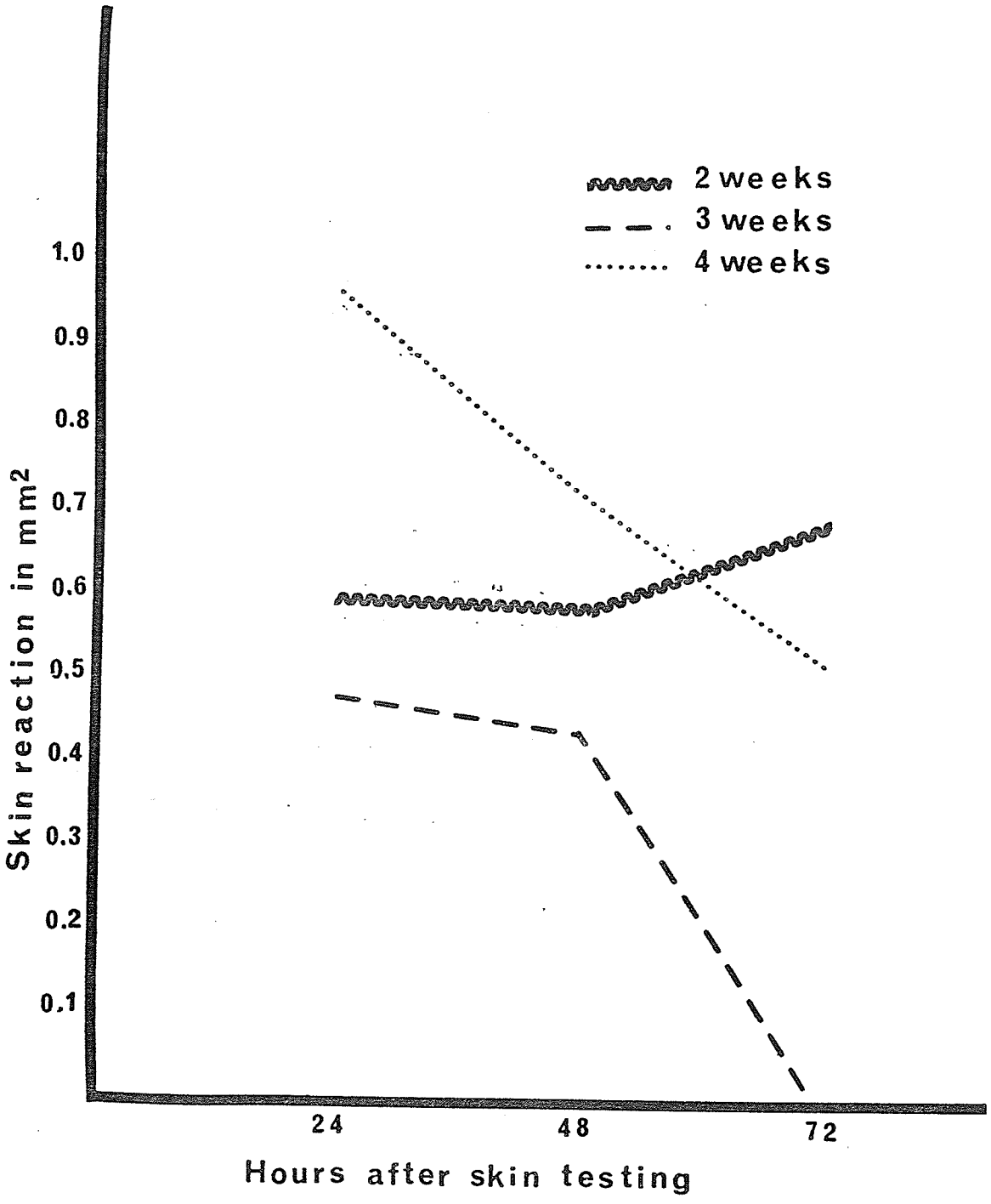

Fig. 1 Skin testing of guine pigs sensitized with LGV agent at 2, 3 and 4 weeks after sensitization

TABLE I

AVERAGE RESULTS OF SKIN TESTING OF GUINEA PIGS OVER A FOUR WEEK TIME PERIOD.

TIME AFTER SENSITIZATION	SKIN THICKNESS (IN MM. ^{2}*) AT		
	24 hr.	48 hr.	72 hr.
2 weeks	0.60*	0.59	0.69
4 weeks	0.97	0.73	0.53

* Verticle axis (mm.) x horizontal axis (mm.) = area of skin reaction.

* Average of five skin measurements.

TABLE II

INDICES OF MACROPHAGE MIGRATION INHIBITION (MI) FROM GUINEA PIGS SENSITIZED WITH INACTIVATED LGV AGENT

ANIMALS	WEEK AFTER SENSITIZATION	AVERAGE AREA* OF MIGRATION IN MM.^{2}		
		INCUBATION WITH Ag*	INCUBATION WITHOUT Ag	MI INDEX**
sensitized	2	0.39	0.63	61.8
sensitized	3	0.47	0.72	65.2
sensitized	4	0.20	0.65	30.7
sensitized	5	0.31	0.85	35.2
sensitized	6	0.45	0.75	60.0
sensitized	7	0.58	0.79	73.5
control		0.93	0.94	99.0

* Ag = antigen.

* Average of five in vitro tests.

** Indices lower than 80 per cent are positive for in vitro cellular inhibition.

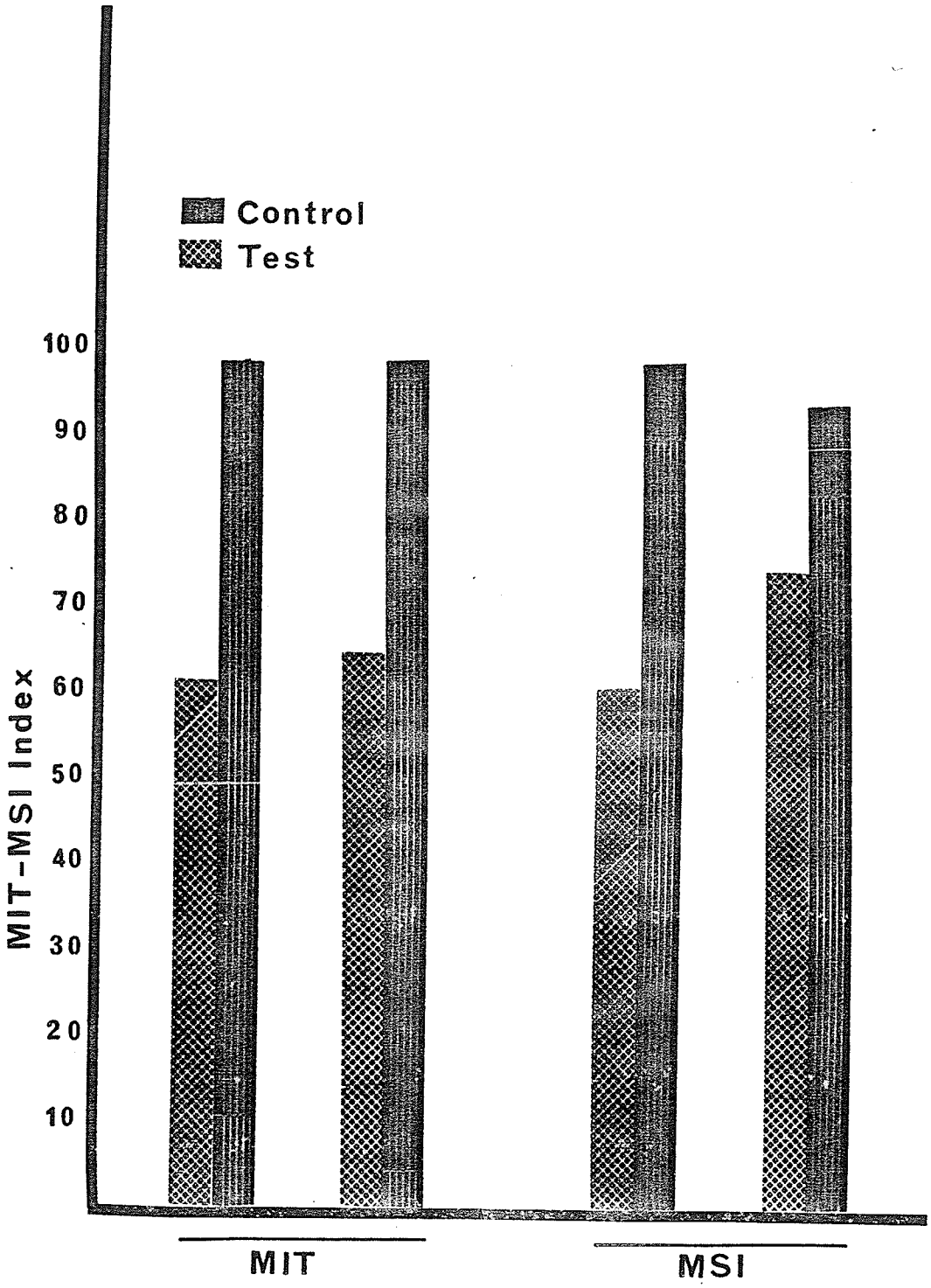

Fig. 2 Comparison of macrophage inhibition test (MIT) and macrophage spreading inhibition (MSI) on mice sensitized with LGV agent

TABLE III

MSI INDICES FROM MICE SENSITIZED WITH INACTIVATED LGV AGENT.

ANIMAL	WEEKS AFTER INJECTION	PERCENTAGE OF CELLS* SPREADING		
		WITH Ag	WITHOUT Ag	MSI INDEX
sensitized	1	14.5	18.7	77.5
sensitized	2	16.0	16.0	50.0
sensitized	3	10.0	16.4	55.5
sensitized	4	16.0	26.0	61.0
sensitized	5	12.1	16.0	75.0
sensitized	6	24.6	33.3	73.9
control		19.5	20.7	94.9

* Figures indicate the average result of five testings.

cent. From the Table it would seem that the optimum sensitization time for mice was 2 to 3 weeks. An arbitrary index of 80 per cent was set as a figure below which sensitization could be considered significant. Above this figure, the degree of sensitization was considered to be negative.

IV. Electrofocusing

A) LKB Electrofocusing Column 8101

The ampholine Electrofocusing column 8101 was used to isolate the various components of the sonicated LGV agent which may be reacting in the cellular immune response. From the graph (figure 3) the fractions were pooled into ten tubes and concentrated by dialysis against 20 per cent polyvinylpyrolidine (PVP) or by evaporation at controlled temperature and ionic concentration. The protein and the non-reducing sugar contents were determined and each of the ten fractions were tested for their MSI indices.

Table IV shows the correlation between the nitrogen and non-reducing sugar content and MSI indices for all ten fractions. The highest MSI reactions were obtained from fractions number 2, 5 and 6. Except for fraction number 2, the nitrogen content is high, with fraction number 5 having 1410 ug. /ml. and fraction number 6 containing 925 ug. /ml. The non-reducing sugar content was high in

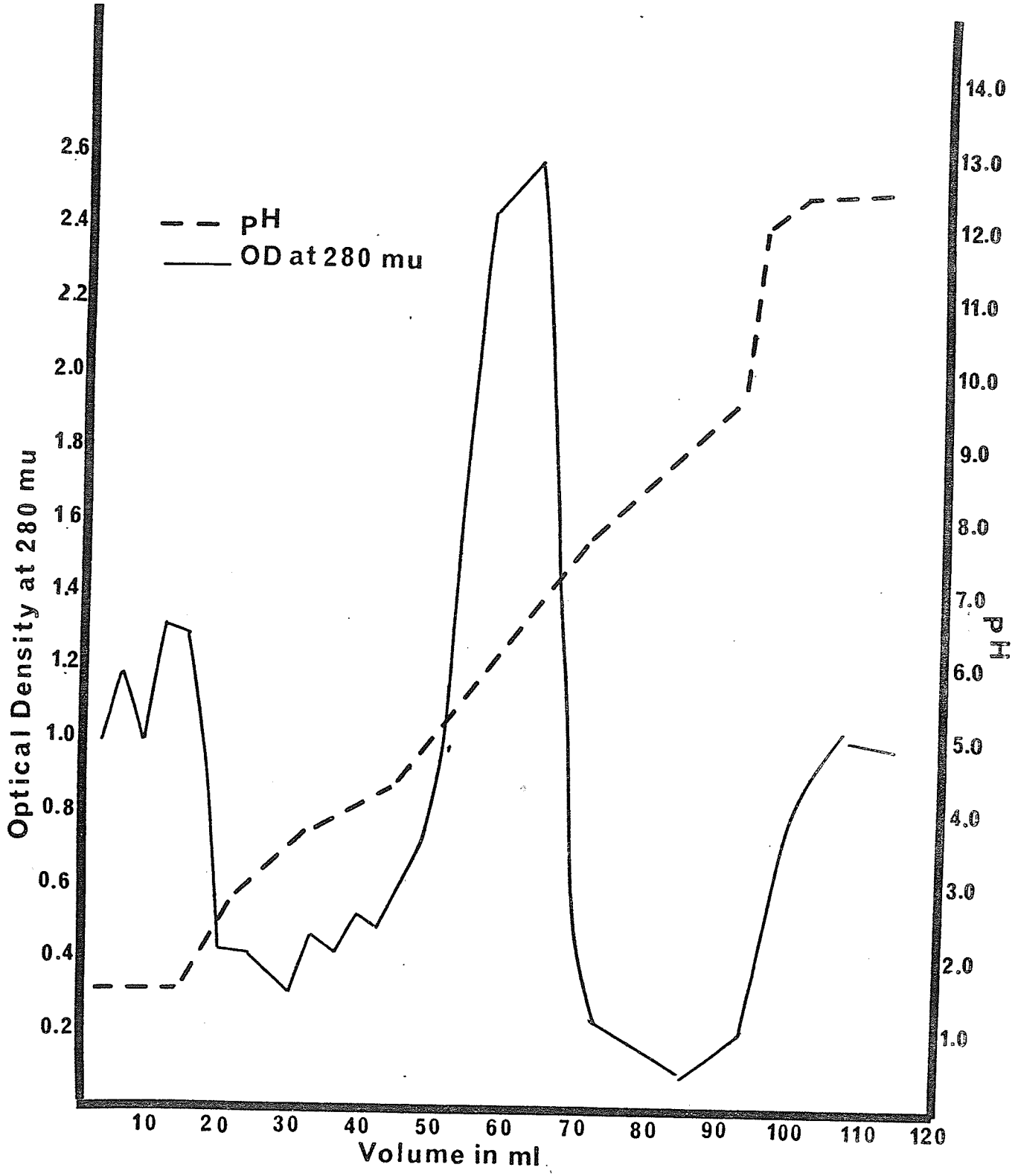

Fig. 3 Fractionation of sonicated LGV agent by electrofocusing using the LKB 8101 column

TABLE IV

CORRELATION BETWEEN NITROGEN AND NON-REDUCING SUGAR CONTENT AND MSI INDEX IN FRACTIONS FROM LKB COLUMN 8101.

NUMBER*	NITROGEN (ug. /ml.)	NON-REDUCING SUGAR (ug. /ml.)	MSI INDEX
1	110	2500	73.5
2	80	2700	56.4
3	1270	6000	93.3
4	2550	7000	78.0
5	1410	5700	51.3
6	925	3900	44.0
7	135	2200	72.0
8	740	4300	94.6
9	295	2900	99.0
10	865	6700	70.5

* Pooled fractions from two columns with comparative isolation patterns.

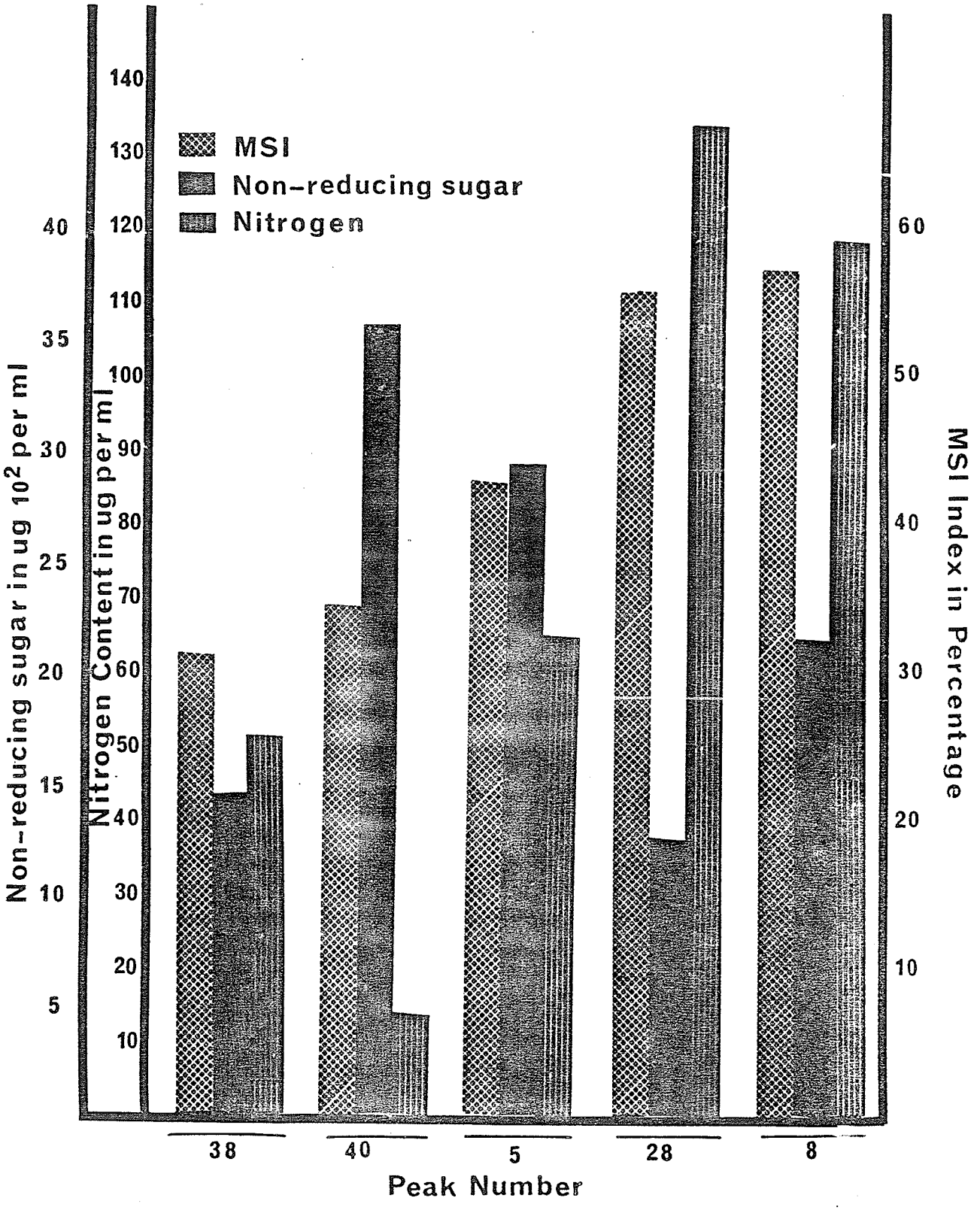

Fig. 4 MSI and chemical nature of LGV fractions obtained from 8102 electrofocusing column

fractions 5 and 6, containing 5700 and 3900 ug./ml. respectively; fraction 2 contained 2700 ug./ml. A comparison of the MSI indices and the biochemical composition can be seen in figure 4.

B) LKB Electrofocusing Column 8102

The LKB Ampholine Electrofocusing column 8102 has a volume of 440 ml. It was decided to use this column in order to obtain a better separation of the components. The column was run similar to the 8101 column. Treatment of the fractions differed only in the method of concentration, i. e., dialysis against 20 per cent PVP only was used and the optical density at 260 mu. was already read. The pattern of elution from this column can be seen in figure 5. It can be seen that the pattern varies greatly from the 8101 column in that more peaks of 280 mu. were obtained. When all the fractions were pooled, 42 peaks were obtained. Each of these peaks was tested for its MSI index. Seventeen out of 42 fractions had MSI indices of less than 80% demonstrating cellular immunity of these fractions. Table V shows these 17 fractions with their corresponding nitrogen and non-reducing sugar content. The peaks with the strongest inhibitory effect on macrophages were 40, 28, 38, 2 and 5 respectively. The nitrogen and sugar content did not seem to form any specific pattern although the fractions with the greater cellular reactivity, i. e.,

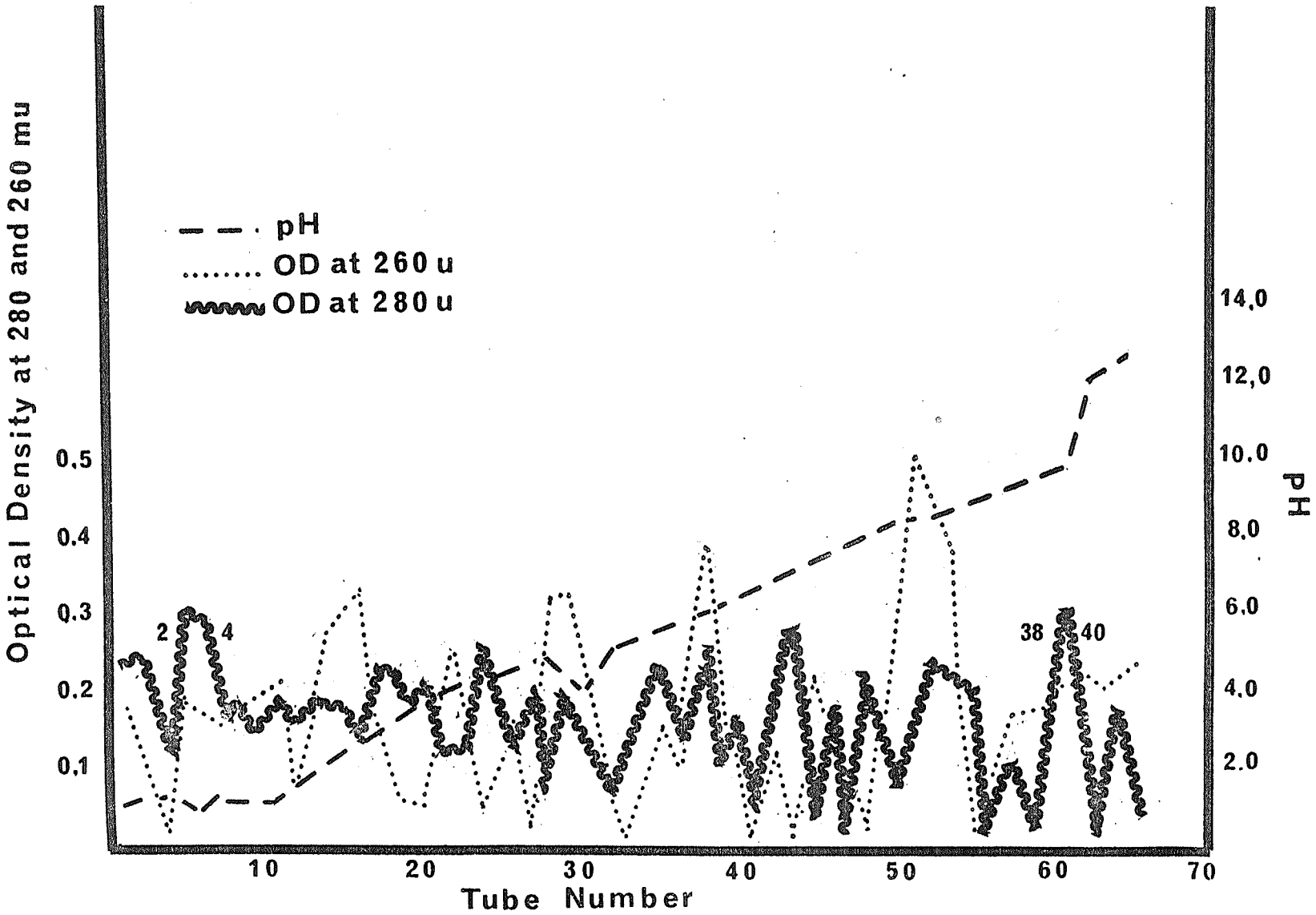

Fig. 5 Fractionation of sonicated LGV agent by electrofocusing using the LKB 8102 column

TABLE V

CORRELATION BETWEEN THE pI, NITROGEN CONTENT, NON-REDUCING SUGAR CONTENT AND MSI INDEX IN FRACTIONS FROM ELECTROFOCUSING COLUMN WITH POSITIVE CELLULAR REACTIVITY.

FRACTION NUMBER	pI	NITROGEN ug/ml	NON-REDUCING SUGAR ug/ml	MSI INDEX*
1	1.25	79	373	60 %
2	1.44	65	255	40
4	1.35	48	400	78.7
5	1.38	65	295	43.1
8	1.48	119	213	57.2
16	4.88	832	130	73.8
18	4.34	332.5	285	59.8
25	6.44	50	62	60.4
27	6.92	23.5	38	69.5
28	7.34	134.1	125	56.5
32	8.15	126	40	64.5
34	8.71	107.9	155	66.3
35	9.08	5	128	78.8
37	9.39	6	214	58.2
38	10.38	51.7	148	31.2
40	12.77	6.75	358	34.7
41	12.98	69	175	65.4
9	2.3	90	740	95.2
11	3.17	132	162	100
17	4.98	187	145	87.5
23	6.15	90.5	113	100
42	12.93	9.5	126	92.5

* MSI indices higher than 80% were considered negative for a cellular mediated reaction.

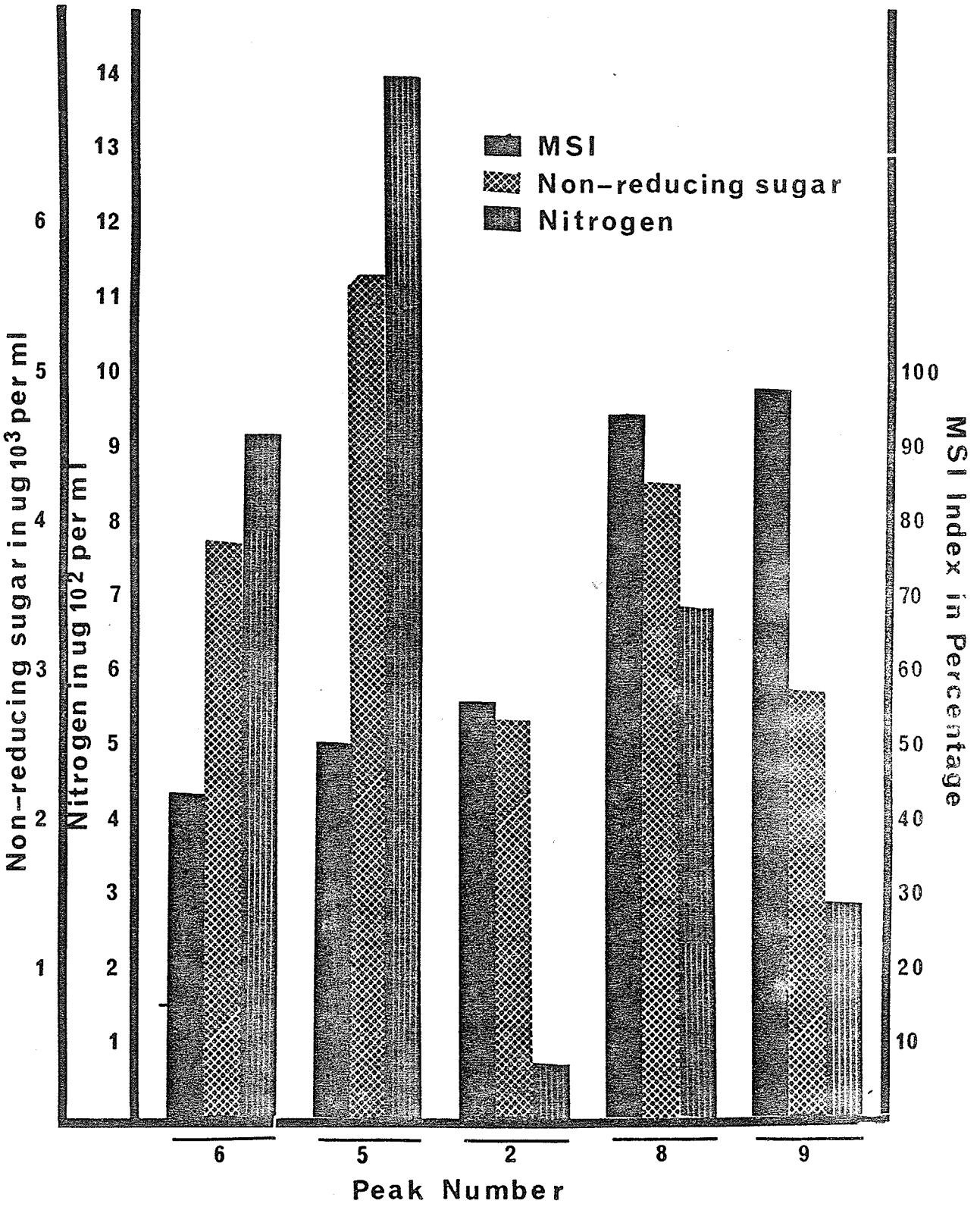

Fig. 6 MSI and chemical nature of LGV fractions obtained from 8101 electrofocusing column

lower MSI indices, seemed to contain lower protein nitrogen content. A comparison of the composition can be seen in figure 6.

V. Disc-gel Electrophoresis

Table VI shows a comparison of the pooled fractions of the two columns by disc electrophoresis. Fraction 2 from the LKB column 8101 corresponded to fractions 2, 4 and 5 of the LKB column 8102. It can be seen that fractions 38, 40 and 41 from the LKB column 8102 did not correspond to any fractions from the 8101 column.

VI. Cytophilic Antibody

Table VII shows the results obtained from the tests for cytophilic antibody from the fractions from column 8101. The lowest index obtained was 91.2% which was well above the 80% value and could be considered as near-normal results, normal being 100%.

TABLE VI

CORRELATION BETWEEN FRACTIONS POSITIVE IN MSI OBTAINED FROM 8101 AND 8102 ELECTROFOCUSING COLUMNS

ELECTROFOCUSING COLUMN		IDENTICAL FRACTIONS (BY IMMUNODIFFUSION AND DISC ELECTROPHORESIS)				
8101	#	2	5	6	9, 10	?
8102	#	2, 4, 5	18	25, 27	35, 37, 42	38, 40, 41

TABLE VII

TEST FOR THE PRESENCE CYTOPHILIC ANTIBODIES ON LGV FRACTIONS PREPARED BY ELECTROFOCUSING.

COLUMN 8101, FRACTION #	MSI INDICES OF CELLS FROM	
	SENSITIZED MICE	NORMAL MICE*
1	73.5	91.7
2	56.4	97.8
3	93.3	97.1
4	78.0	95.6
5	51.3	92.8
6	44.0	95.0
7	72.0	94.0
8	94.6	94.8
9	99.0	95.9
10	70.5	91.2

* Cells from sensitized animals were incubated at 56°C for 30 minutes, centrifuged and supernatents were added to cells from non-sensitized animals. The latter were tested for inhibitory effect of different fractions.

DISCUSSION

The results obtained from the skin tests followed what was already known about the psittacosis dermal reaction (Kilham, 1948). The Frei test (1925) has been used for diagnostic purposes. A raised papule 6 x 6 mm. was considered to be a positive reaction if the control was 5 x 5 mm. or less. Clinical symptoms were used in conjunction with the skin test as false positive dermal reactions were demonstrated in patients with tuberculosis, septicemia, fever, or co-existant early syphilis. In some cases of clinically diagnosed LGV, the dermal reaction was positive while a negative complement fixation test was obtained (Meyer, 1965). The mechanism underlying the skin reactions has not been fully identified although it was thought to be a delayed sensitivity reaction because of the histological picture of the disease. As the LGV skin reactions are more widely used than those of the other chlamydial agents and the material more easily available, LGV was used for this investigation. The purpose of this study is:

1) to establish the presence of cellular mediated sensitivity to chlamydial infections manifested by skin reactions through using an in vitro cellular reaction.

2) to correlate the in vitro cellular activity with the dermal reaction.

3) to characterize the component or components responsible for the delayed reaction.

The first step was to investigate the dermal reaction and time of sensitization required for its manifestation. Table I shows the results of such tests. While a reactivity was found after two weeks of sensitization, this reactivity was increased with the period of sensitization. The results correlate with what was already known about LGV skin reactivity (Kilham, 1948). That is, that the reaction is delayed in onset and remains for several days, in our case, a reaction was still visible after 72 hours. This was one of the criteria for differentiating delayed sensitivity from the immediate reaction (Sell and Asofsky, 1967). As in all cellular immune reactions the degree of reactivity depends on the extent of sensitization of the animal among other factors. A mechanical factor, such as the depth of the antigen in the skin will affect the extent of the cutaneous manifestation.

For more accurate quantitative analysis of the cellular immune response, the in vitro tests were used. The two methods were the MI and MSI tests (David et al., 1964; Fauve and Dekaris, 1968). Positive in vitro results, correlated with the skin reaction, would show that the chlamydial agents were capable of eliciting a cellular immune reaction in their hosts. It can be seen from our result that there is a definite cellular response that increases to a maximum reactivity after 3 to 5 weeks of sensitization then gradually decreases. Figure 2 shows the height of reactivity of the

guinea pig to be four weeks using the MI test; and of the mice to be three weeks using the MSI test. The slight variation is dependent on the test animal and the in vitro method performed. The slightly varied immune mechanisms of each animal would also affect their immune responses. It can be assumed that the maximum cellular response is reached in the sensitized guinea pig in four to five weeks, and in mice is two to three weeks. In each sensitized animal, a gradual decrease of reactivity occured after the maximum reactivity was demonstrated.

Many variables will affect the results of the MI, such as pH, temperature and most important, the condition of the cells in regards to their metabolic activity. Cells that had not been thoroughly washed of the oil used to increase the macrophage population in the peritoneal cavity were not suitable for testing by the MI test. Such cells did not migrate to any extent in the absence of the antigen and there was no change in the pH of the incubating medium after 24 hours incubation. Cell lysis also occured, as the level of macrophages in the capillary tube lowered during the incubation period. Due to the presence of a number of variables mentioned above, the MI test was an exacting and lengthy method. For these reasons, the MSI was examined as a suitable test for measuring in vitro cellular sensitivity.

The time period involved for the actual MSI test was minimal and many factors which limited the use of MI test were eliminated. For

example, the methods of handling the cells were less exacting and less strenuous on the viability of the cells. Because oil was not used, extensive washing of the cells was not required and the cells could be used for the MSI after washing three times with tissue culture medium 199. One drawback to this method is examination of the macrophages under phase-contrast microscopy and in differentiating between spreading and non-spreading cells. However, Fauve and Dekaris (1968) showed that their results were reproducible by different experimenters, and with experience this factor can be overcome. Macrophages were considered to have spread only when the cytoplasm was clearly visible and the cell is showing irregular borders. At the same time the spreading macrophages appeared darker.

Correlating the results of the MI and MSI tests, it was thought that a cellular mediated immunity was operating in LGV infections and perhaps for other chlamydial agents since these agents share antigens which elicit skin reactions in sensitized hosts (Bietti, 1967). This leads to the speculation as to the nature of the component or components active in the cellular mediated immunity which might be also shared by other chlamydial agents, i. e., a group antigen.

For the purpose of separating the components of the sonicated LGV agent, the LKB Electrofocusing technique was used. The smaller 8101

column, showed three peaks (figure 3) which were divided into ten fractions for analysis. Table IV shows that fractions 2, 5 and 6 of the pooled eluates had the lowest MSI indices and consequently inhibited migration of macrophages the most. This meant that these fractions contained a component or components which were reactive with sensitized cells and, therefore, active in the cellular immune response of the host. The biochemical composition of these fractions did not show any trend in component composition, except to suggest a possible protein-carbohydrate complex, the fixed ratio of which can not be determined from this study (figure 4).

A more extensive separation was necessary in order to affect a more complete separation of the agent. The larger LKB Electrofocusing column 8102 was used. The pattern of separation of the sonicated agent can be seen in figure 5 where a total of 42 fractions were obtained. Of these, 17 demonstrated MSI indices less than 80% as shown in Table V, indicating a cellular inhibition that was significant. These 17 fractions obtained from the 8102 column were compared to those obtained from the 8101 column. Several more active complexes were isolated by using the larger column and were not detected in eluates of the smaller column. However, the ratio of protein to non-reducing sugar in the eluates was not different when the two columns were compared and again, no trend in composition was demonstrated (figure 6).

From these fractions, no concrete idea of the antigenic composition could be demonstrated. However, it is possible to speculate that the reactive antigens in vivo are small protein or peptide molecules associated with a carbohydrate carrier. This protein-carbohydrate complex may be the inducer of the hypersensitive state in vivo, as it has already been shown that they will react in the in vitro tests. While proteins have been known to be excellent inducers of the delayed reaction, polysaccharides alone have failed to produce the same result. Gerety et al. (1970) showed that a very weak delayed reaction was produced with a purified polysaccharide of Diplococcus pneumoniae PnII SSS. For the induction of this state complete Freund's adjuvant was required. It is the configuration of the antigen that plays a large role in the immune response. For instance, David et al. (1964) showed that a protein carrier with a DNP hapten were both necessary for identification by the cells. However, the carrier was more important than the hapten. The difference in configuration between a carbohydrate and protein molecule may account for the differences in immune reactions. A protein moeity with a carbohydrate carrier as pictured by the results presented here would be fairly effective in eliciting a cellular mediated immunity.

In studies of delayed sensitivity using vaccinia virus, it was found that the coat antigens were more reactive in the in vitro tests than the

intracellular antigens (Uedo and Nozima, 1969). It is possible that such is the case for chlamydial agents. However, here, two forms of the LGV agent are usually present, the elementary body and the initial body. Whether different antigens are present in these bodies and which of these will induce the cellular mediated immunity is not known.

The fractions from the two electrofocusing columns were compared by disc electrophoresis to determine which fractions correspond to each other from the two columns. This comparison can be seen in Table VI where only fractions 38, 40 and 41 from the large column did not correspond to any other fraction from the smaller column. There are several reasons for this finding. While the three fractions migrated and were separated on the larger column, the lack of space may have caused them to be denatured on the smaller column and could not be detected in their proper position. Also, being at the extreme ends of the pH gradient might affect the isolation of some components in separate fractions. However, from Table V it can be seen that fractions 38 and 40 show a high degree of reactivity in the in vitro testing.

Several researchers have implicated the presence of cytophilic antibodies as a mechanism behind the delayed sensitivity (Amos et al., 1967; Heise et al., 1968). However, David et al. (1964) in his original studies was not able to detect the presence of cytophilic antibody in his system.

Our LGV system was tested to see whether cytophilic antibodies could be detected. Table VII shows the results of these experiments using fractions from the electrofocusing 8101 column. It can be seen that the supernatants from sensitized cells had a minimal effect on the normal macrophages. They reacted as normal macrophages would be expected to react showing MSI indices similar to non-sensitized cells. Thus, it would seem from these experiments that a cytophilic antibody is not present on macrophages from animals sensitized with the LGV agent. This is in contrast to the work of Heise and Weiser (1968, 1969) in experiments with protein production by RNA which affects cell migration. They postulated that the complex produced by RNA was a gamma G antibody acting as the cytophilic antibody. However, the pathogenicity of chlamydia is not well understood, and it might be possible that other factors are responsible for cytophilic antibody not being detected, if indeed, it is present in this system. We have not tested for cytophilic antibodies in the sera of the sensitized animals.

From viewing the results of the skin tests and the in vitro reaction, a true cellular immune sensitivity is present in LGV infections. However, no light has been shed on the mechanism behind the response or the manner by which the response is induced. The correlation of the dermal reaction and the cellular reactivity has helped to a certain extent, but more is needed in order to understand the role of cellular immunity in protection

against chlamydial infection. The humoral reactions were shown not to be related to the delayed reactions (Barwell, 1952; Meyer, 1965). Hirsh et al (1968) found that the use of antilymphocyte serum affected the cellular immune response of vaccinia virus, but had no effect on the primary and secondary reactions. There was also a higher morbidity and mortality rates in animals injected with the antilymphocyte serum, suggesting a type of protection conferred on the animal by the cellular mediated immunity.

Further work in this field would be desired to clarify the role played by delayed sensitivity in the body defense mechanisms to chlamydial agents. The component or components reactive in the in vitro tests should be more closely examined for their composition and relation to the host. The MSI is a method by which members of the chlamydial group of agents can be studied for the relationship between these agents, as it is known that one of the group specific antigen is a skin test antigen (Meyer, 1965). All of this information might be also of help in solving the puzzle of the pathogenicity of chlamydial agents.

APPENDIX

Eagle's Minimal Essential Medium

1.07	gm. MEM powder (BDL)
0.035	gm. $NaHCO_3$
85.0	ml. distilled water
15.0	ml. normal guinea pig serum

The powder was added to the 35.0 ml. of distilled water and the 0.035 gm. of $NaHCO_3$ was added. The pH was adjusted to pH 7.0 - 7.2 and the solution was sterilized by Millipore filtration. Fifteen ml. of normal guinea pig serum was added to make up a 15% concentration.

Medium 199

10.0	gm. powdered medium 199 (BDL)
20.0	mg. Bovine serum albumin
5.000	units heparin (5 units/ml.)
2.2	gm. $NaHCO_3$
950.0	ml. distilled water

The powdered medium was added to the distilled water at room temperature without heating. The BSA, heparin and $NaHCO_3$ was added and the solution stirred. The volume was then made up with distilled water to a 1000 ml. volume. The pH was adjusted to 7.0 - 7.2 and the medium sterilized by Millipore filtration.

LKB Electrofocusing Ampholine Solutions

Electrofocusing column 8102

Dense electrode

0.8	ml. H_2SO_4
56.0	ml. distilled water
48.0	gm. sucrose

The acid was added to the distilled water, then the sucrose was added. The solution was stirred constantly until the sucrose was dissolved.

Light Electrodes

0.4	gm. NaOH
40.0	ml. distilled water

The NaOH was dissolved in the distilled water.

Dense Ampholyte

7.5	ml. 40% ampholyte - pH 3 - 10
150.0	ml. distilled water
100.0	gm. sucrose

The sucrose was added to the ampholyte-distilled water solution and stirred until the sucrose was dissolved.

Light Ampholyte

2.5	ml. 40% ampholyte pH 3 - 10
215.0	ml. distilled water

The ampholytes were added to the distilled water.

Electrofocusing column 8101

Dense ampholyte

42.0 ml. distilled water

28.0 gm. sucrose

The sucrose was dissolved in the distilled water and 1.5 ml. of 40% ampholytes (pH 3 - 10) was added to 48 ml. of the sucrose solution.

Light ampholyte

49.0 ml. distilled water

0.6 ml. 40% ampholytes pH 3 - 10

The ampholytes were added to the distilled water and mixed well.

Dense electrode

0.2 ml. H_2SO_4

14.0 ml. distilled water

12.0 gm. sucrose

The solution was made up according to the directions for the dense electrode of column 8102.

Light electrode

0.1 gm. NaOH

40.0 ml. distilled water

The NaOH was dissolved in the distilled water.

Disc-gel Electrophoresis

Anion Gel System

A. Lower Gel

a)	Acrylamide	30.0 gm.
	Bisacrylamide	0.8 gm.
	Distilled water	to volume of 100 ml.
b)	Tris	18.15 gm.
	1.0 NHCl	24.0 ml.
	Temed	0.24 ml.
	Distilled water	to volume of 100 ml.
c)	Ammonium persulphate	0.14 gm.
	Distilled water	to 100 ml.

Before use, a, b and c are added together in a ratio of 1:1: 2 respectively.

B. Upper Gel

a)	Acrylamide	10.0 gm.
	Bisacrylamide	0.8 gm.
	Distilled water	to 100 ml. volume
b)	Tris	2.23 gm.
	1 M H_3PO_4	12.8 ml.
	Temed	0.1 ml.
	Distilled water	to 100 ml. volume

c)	Riboflavin	2.0 mgm.
	Distilled water	to 100 ml. volume
d)	Ammonium persulphate	80.0 mgm.
	Distilled water	to 100 ml. volume

Before use, a, b, c and d were mixed in a ratio of 1:1:1:1 respectively.

C. Upper Buffer

Tris	5.16 gm.
Glycerine	3.48 gm.
Distilled water	to 1000 ml. volume

D. Lower Buffer

Tris	14.5 gm.
1 N HCL	60.0 ml.
Distilled water	to 1000 ml.

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