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CELL-MEDIATED IMMUNE RESPONSE IN SHEEP RE-INFECTED WITH ORF VIRUS

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SUMMARY

Sheep experimentally re-infected with orf virus developed an accelerated skin reaction and exhibited a cell mediated immune (CMI) response, as determined by leucocyte migration inhibition (LMI) tests and lymphocyte transformation (LT) tests. The CMI response was detected day 4 post scarification using the direct LMI tests. It persisted through day 63 with maximum response occurring 15 days post challenge. Measurement of CMI in the re-infected sheep by LT, however, was evident on the fifth day and lasted 42 days post challenge.

INTRODUCTION

Although 'solid' immunity was believed to develop in sheep after natural orf virus infection or vaccination (Carr'e, 1932; Glover, 1928; Jacotot, 1926; Nisbet, 1954), some antithetical views have recently been expressed which led to more investigation into the nature of the immunity. Firstly, most recovered or vaccinated sheep reacted to secondary challenge with an accelerated response. Normally in a primary reaction. The lesions resolve 38 to 40 days after infection but in secondary reactions the lesions take only 10 - 14 days to resolve (Boughton and Hardy, 1934; Osman, 1976; Schmidt, 1962). Secondly protection from humoral antibodies seems to be weak or ineffective. Attempts to passively transfer resistance to susceptible sheep using serum from hyperimmunized or recovered sheep have always failed (Khanduer *et al.*, 1969). Thirdly, it has been reported that lambs from 'immune' sheep did not acquire protection from colostral antibodies (Kerry and Powell, 1971). Moreover, vaccination which has been practiced for many years seems to have had little effect on the endemicity of the disease (Scott; personal communication). Hence, the possibility that immunity to orf might be due to factors other than humoral responses was investigated. Aynaud (1923) was the first to postulate that cell mediate immunity could be involved since he failed to demonstrate neutralizing antibodies in sera from convalescent sheep. Later Osman (1976) attempted to prove this hypothesis by transferring resistance to susceptible lambs using lymphoid cells from sheep which had just recovered from orf. The results he obtained were not conclusive. Thus, this project was proposed to study the involvement of CMI using migration inhibition tests and lymphocyte transformation tests.

MATERIALS AND METHODS

The Virus and its Antigen

The strain of orf virus used was collected from naturally occurring cases in sheep in the Easter Bush Farm, Roslin, Scotland. Twenty per cent orf virus particles suspension was prepared using scabs from lesions in phosphate buffered saline (PBS).

Animals

Suffolk and cheviot breeds of sheep and/or their crosses were used. Majority were weaners but older and younger lambs were used in some experiments. Some sheep were designated susceptible if they came from a flock in which the animals had no apparent signs of orf after 10 years of clinical examination and these acted as controls. Sheep designated as previously infected were those in which the disease had run its full course after natural or experimental infection period.

Experimental Infections.

Sheep were scarified on the inner side of one of the thighs using sterile disposable blood lancets and then 0.1 ml of the 20% scab virus suspension was applied. Twenty previously infected sheep were challenged with orf virus by scarification and sixteen others were not infected and acted as controls. Blood was collected once a week in heparinized vacutainers from the challenged and control animals. Similarly, on days 0, 10, 15, 20, 42 and 63 post-challenge blood was collected and lymphocytes for LT tests harvested. The lesion development was checked daily in the infected sheep.

Indirect Migration Inhibition Test

The indirect migration tests were carried out using normal peritoneal exudate (PE) cells from guinea pigs to assay the migration inhibition factor (MIF) and leucocytes from normal sheep to assay leucocyte inhibition factor (LIF) in a cell-free supernate obtained from lymphoid cell cultures of sheep infected with orf virus. Lymphocytes were harvested from the popliteal and prefemoral lymph nodes, the spleen and the thymus from two sheep re-infected with orf virus by squeezing cut and teased pieces from these organs separately through a sieve with a mesh of 200. The cells were washed four times in hanks buffered saline and their viability and total count determined by the dye exclusion method using 0.3 percent Nigrosin dye. A suspension of 3.5 million cells/ml was made in growth medium consisting of RPMI 1640, hepes buffer, 10 per cent heat inactivated foetal calf serum plus 100 units of penicillin and 100 mg of streptomycin. The cell suspension was then distributed into tissue culture bottles and the concentration, determined by checker board titration method, cell-adapted orf virus was added to some bottles; the rest were left as controls. The cultures were then incubated at 37°C for 24 hours. After which the cell-free supernatant was harvested and concentrated to 50% original volume by dialysis against 40% polyethyleneglycol. The concentrated supernatant was dialyzed against RPMI 1640, then replenished with 10% heat inactivated foetal calf serum and antibiotics before storage at -20°C.

Peritoneal exudate (PE) cells rich in macrophages were induced in two guinea pigs by injecting them intraperitoneally with a mixture of PRMI 1640 medium and incomplete Freunds adjuvant. The PE cells were harvested by injecting warm Hanks balance salt solution with heparin (HBSS-H) into the peritoneal cavity and gently kneading the abdomen and collecting the cell-rich HBSS-H with a 20ml syringe. The cells were washed in RPMI 1640 medium, checked for viability, then suspended in the RPMI growth medium at 10^7 cells/ml concentration. The cell suspension was drawn into sterile capillary tubes. The tubes were sealed at one end with cristaseal then centrifuged at 2,000g for five seconds. The tubes were then cut at the cell-fluid interface and the end containing cells fixed in a chamber of the migration plate. Concentrated cell-free supernatant fluid was then added to each chamber after warming it to 37°C in a waterbath. The chambers were sealed with coverslips and the whole plate incubated at 37°C in atmosphere of 5% CO_2 for 24 hours. Control chambers contained the

supernatant fluid from the lymphocyte cultures into which orf-virus was omitted. Similarly, leucocytes harvested from 10ml of heparinized blood from normal sheep which had no history of orf infection were washed and processed in the same way as the PE cells to assay the presence of LIF in the cell-free supernatant fluid. The image of the tube and the area of migrating cells was viewed by photo-enlarger and thereby projected on to graph paper. The outline of the cell migration was determined by counting the millimeter squares within the outline. The results were expressed by the migration index (MI) obtained from the equation:

MI = (Area of migration of cells with lymphokine X 100)/(Area of migration of cells without lymphokine).

Direct capillary leucocyte Migration Inhibition Test

Blood for leucocyte separation was collected into heparinized vacutainers from sheep either challenged with orf virus by scarification or unchallenged sheep as controls. The erythrocytes were haemolysed by using sterile distilled water and the rest of the cells restored to isotonicity within 30 seconds by the addition of 3.5% sodium chloride solution. The leucocytes were then processed as the PE cells and put on to chambers. In the chambers 0.1 of 10^{-2} virus dilution was added and in the control chambers only growth medium.
The area of migration of the leucocytes was determined in the same way as for the indirect test and results were expressed as follows:

MI= (Area of migration of leucocytes with antigen X 100)/(Area of migration of leucocyte without antigen)

Lymphocyte transformation Assay

Sheep lymphocytes were separated from buffy coasts using 'lymphoprep. The lymphocytes, which form a white band at the interface of saline and 'lymphoprep' were carefully pipetted into sterile siliconized tubes and washed three times in HBSS-H. Viable cells were counted by Nigrosin dye exclusion method and then suspended in RPMI- 1640 growth medium at a concentration of 10^6 cells/ml.
The lymphocyte suspension was distributed into tissue culture micro-plate at the rate of 0.2 ml per well. The stimulant (PHA or 10^{-2} was added as predetermined. All cultures were set in triplicate at 37°C in a humidified atmosphere of 5% CO_2 for five days. Controls included were PHA as a positive control and growth medium instead of PHA or orf antigen to non-stimulated cultures.
Blastogenesis was determined by pulsing the cultures with titrated thymidine of 5 Ci/mmol, specific activity at 2uCi per well, 24 hours before terminating the culture with cold PBS (pH 7.3). The cultured and labelled lymphocytes were washed twice with chilled PBS and then precipitated with 6% trichloracetic acid aqueous solution overnight at 4°C. The precipitate was dissolved in NCS solubilizer, a quaternary ammonium base in toluene, and then mixed with 5 ml of the scintillating cocktail; Radioactivity, expressed counts per minute (CPM) was measured in a scintillation Beta counter. 3H thymidine uptake results were expressed as stimulation index (SI) calculated as follows:

SI = (CPM of cultures with orf antigen or PHA)/(CPM of cultures not stimulated with any antigen).

Two standard phosphorus = PPO and POPOP

RESULTS

Clinical Response.

There was no obvious systemic response in the sheep following the application of orf virus

suspension to scarified areas. The local reaction which occurred evolved through the following stage. On second and third day post-challenge
there was reddening and oedema along the lines of scarification. This was followed by papules and vesicles on the 4th to 6th day, pustules on the seventh day and scab development on 10th day post-challenge. Complete resolution in the re-infected sheep took two to three weeks. Unchallenged control animals remained normal during the study.

Migration Inhibition Tests (MIT)

When the indirect MIT and LMI were performed, sensitized lymphocyte cultures inoculated with orf virus antigen in vitro, produced lymphokines MIF & LIF, respectively that inhibited migration of macrophages (PE cells)
and PMN leucocytes (Fig 1 & 2). The CMI response as evaluated by LMI tests was detected in sheep re-infected with orf virus (Fig. 3 & 4). In 14 previously infected sheep which were challenged with orf virus, the Migration index (MI)before challenge was 79.0% indicating that the animals possessed some sensitized lymphocytes due to previous exposure to the virus. The indices decreased after challenge to minimum value of 45%, fifteen days post-challenge, and were maintained at about 50% for at least seven weeks (Fig. 3). Likewise, the MI obtained from the previously infected sheep which were not challenged (Control group) was 66.4% and fluctuated between 66 and 85% during the duration of observation. They were always below 100% indicating presence of some sensitized lymphocytes from previous exposure to the virus (Fig. 3). Comparing results of the challenged previously infected sheep with those of the unchallenged previously infected sheep a significant difference was observed from day 4 to day 63 (Fig. 4; Table 4).

Lymphocyte Transformation Tests

Lymphocytes derived from sheep which recovered recently from the viral infection incorporated more radioactive thymidine when cultured with orf-virus antigen compared to similar cells cultured in absence of the antigen (Table 1). The antigen at various dilutions significantly stimulated uptake of the thymidine by the lymphocytes. Maximum thymidine uptake was observed for lymphocytes harvested by day 4 and 5 post infection (PI) in response to phytohaemagglutinin mitogen and orf viral antigen stimulation respectively. The maximum response was 13,993.67 and 1393 mean counts per minute (CPM) for the mitogenic and antigenic (10^{-2} dilution) stimulation respectively. Lymphocytes similarly cultured without a stimulant (control) showed a mean maximum of 464C=PM. There was no increase in uptake of the radioactive thymidine by lymphocytes from susceptible lambs. The maximum CPM was 390 and 443 for lymphocytes cultured with and without orf antigen respectively (Table 2). The results of the uptake of radioactive thymidine by orf stimulated lymphocytes from previously infected challenged sheep were expressed by SI values (Table 3, Fig.5). The means of these values increased 1.06 on day 0 to a maximum of 4.34 day ten after re-infection, then followed by a gradual decline to pre-challenge values nine weeks later.In contrast, the Si-values for the unchallenged group fluctuated between 0.96 and 1.4 throughout the study period and were significantly different from those of the test sheep from days 5 to 42 after challenge (Table 3, Fig.5). The blastogenesis detected in the PHA-stimulated lymphocytes was consistently higher than that detected in the orf antigen lymphocytes or non stimulated lymphocytes.

Figure 1: The migration of cultured PE cells from guinea pigs. (a) Cell - free supernate from sensitised lymphocyte cultures stimulated by orf virus. (b) Cell - free supernate from sensitised lymphocyte cultures not stimulated.

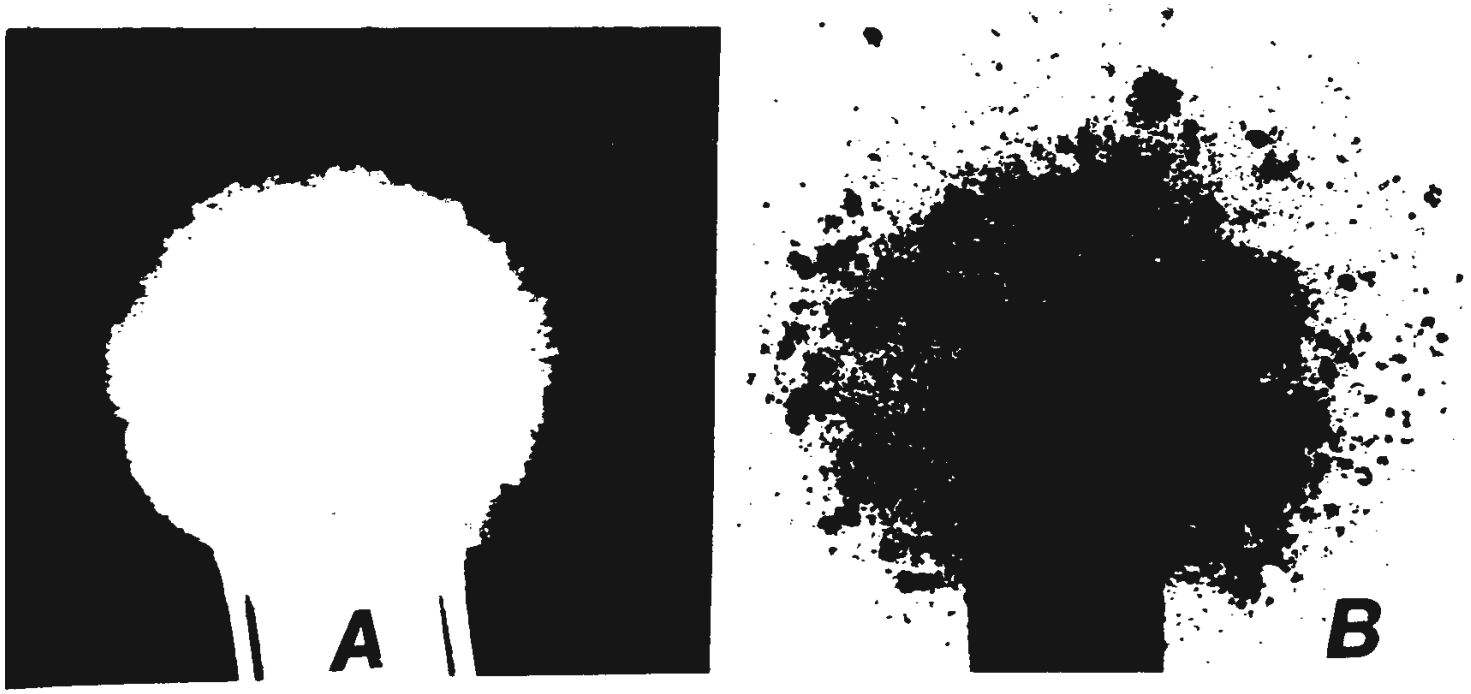

Figure 2: The migration of culture leucocytes from a normal 'susceptible' sheep. (a) Cell - free supernate from sensitised lymphocyte cultures stimulated by Orf virus (b) Cell - free supernate from sensitised lymphocyte culture not stimulated.

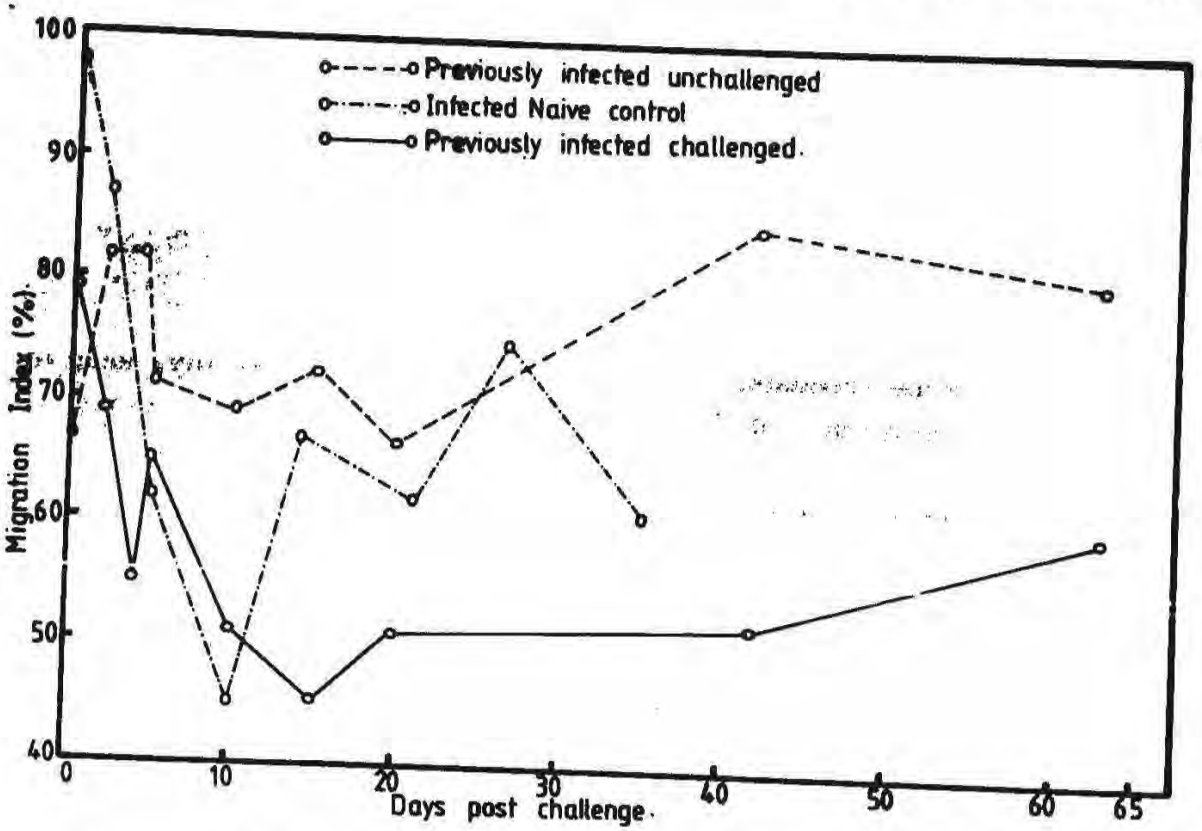

Figure 3: Migration indices of sheep re-exposed to virus and naive control

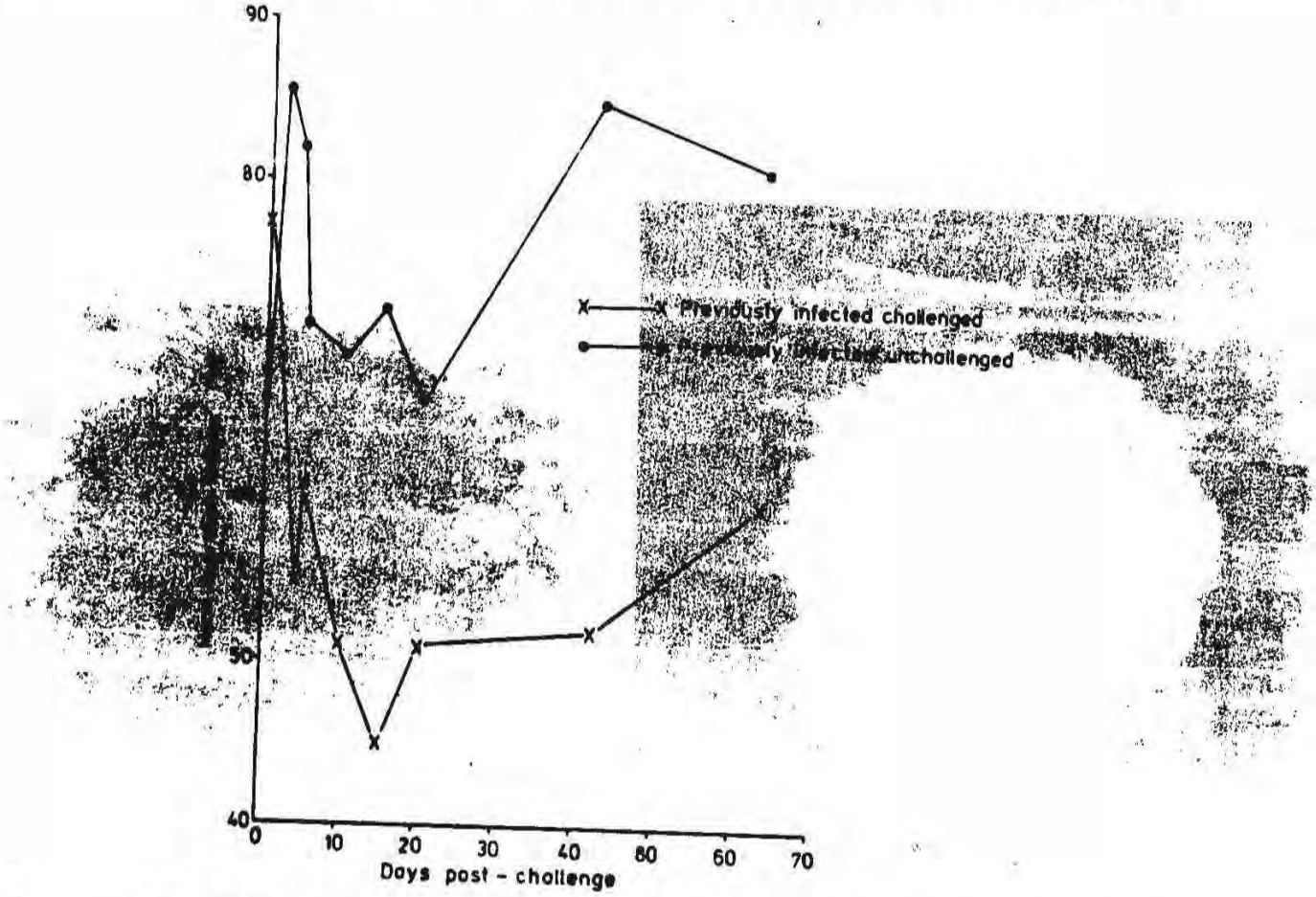

Figure 4: Mean leucocyte migration indices of previously infected sheep re-challenged and unchallenged control.

Table 1: Uptake of radioactive thymidine by Orf virus antigen primed lymphocytes serially taken in from sheep infected with sa virus (Mean counts per minute ± standard deviation)

Days post-inoculation when cells were harvested	SOURCE OF STIMULANT						
	ORF antigen*				PHA**	CONTROL	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-10}	0.5ul/ml	No. St.
2	372.3 ± 43	360 ± 23	606 ± 234	535.8 ± 6	357.3 ± 12	7011.2 ± 4422	407 ± 159
3	556 ± 88	398 ± 290	818.75 ± 219	471.25 ± 34	295.67 ± 60	8662.67 ± 4505	397.67 ± 86
4	750.5 ± 63	481.5 ± 34	739.5 ± 438	609.5 ± 65	313.75 ± 20	13993.67 ± 2062	451 ± 428
5	1130 ± 98	1393 ± 170	1263 ± 89	1037 ± 141	566.75 ± 90	6858.3 ± 847	464 ± 140
6	472.67 ± 155	522.2 ± 156	429 ± 130	538.8 ± 175	425.5 ± 56	2439.8 ± 341	390 ± 68
7	496 ± 301	523.3 ± 197	538 ± 286	432.3 ± 131	521.5	8875.5 ± 283	461 ± 125

* Serial dilution ** Phytohaemagglutimin

Table 2: **Uptake of H3 Tdr by non-sensitive lymphocytes from a susceptible control group**

Sheep	PHA-MITOGEN	ORF-ANTIGEN	WITHOUT ANTIGEN	ANTIGEN SI=Control
134	1405.5±630	145.25±27	187±3	0.78
144	2414±112	390.5±26	443±34.2	0.88
145	2446.5±118	291±17.8	2236±38	1.23
147	2615.5±2306	189±127	190±0	0.99
148	2813±193.6	356±54	414.5±17.5	0.86

Table 3: **Comparison of the mean SI Values between the challenged and unchallenged previously infected sheep.**

Days Post Challenge	d.f	S. I. Challe-nged	Value Unchal-lenged	t	p	Inter-preta-tion
0	10	1.06	0.96	0.9	0.400	N.S.
5	10	2.66	1.07	5.29	0.001	H.S.
10	8	4.34	1.04	7.91	0.001	H.S.
15	10	1.96	0.96	6.62	0.001	H.S.
20	10	2.60	0.98	4.89	0.001	H.S
42	10	1.36	1.05	3.74	0.010	H.S.
63	10	1.08	1.14	1.22	0.300	H.S

H.S. = Highly Significant
N.S. = Not Significant

Table 4: **Comparison of the daily means of migratory indices between challenged previously infected sheep.**

Days post challenge	D.f	t	p	Interpretation
0	22	1 51	0.200	N.S.
2	6	1.66	0.200	N.S.
4	6	3.09	0.050	S.
5	12	0.69	0.600	N.S.
10	20	3.12	0.010	H.S.
15	18	5.48	0.001	H.S.
20	12	3.31	0.010	H.S.
42	12	7.15	0.001	H.S.
63	14	2.89	0.020	S

S. = Significant.
H.S. = Highly Significant.
N.S. = Not Significant.

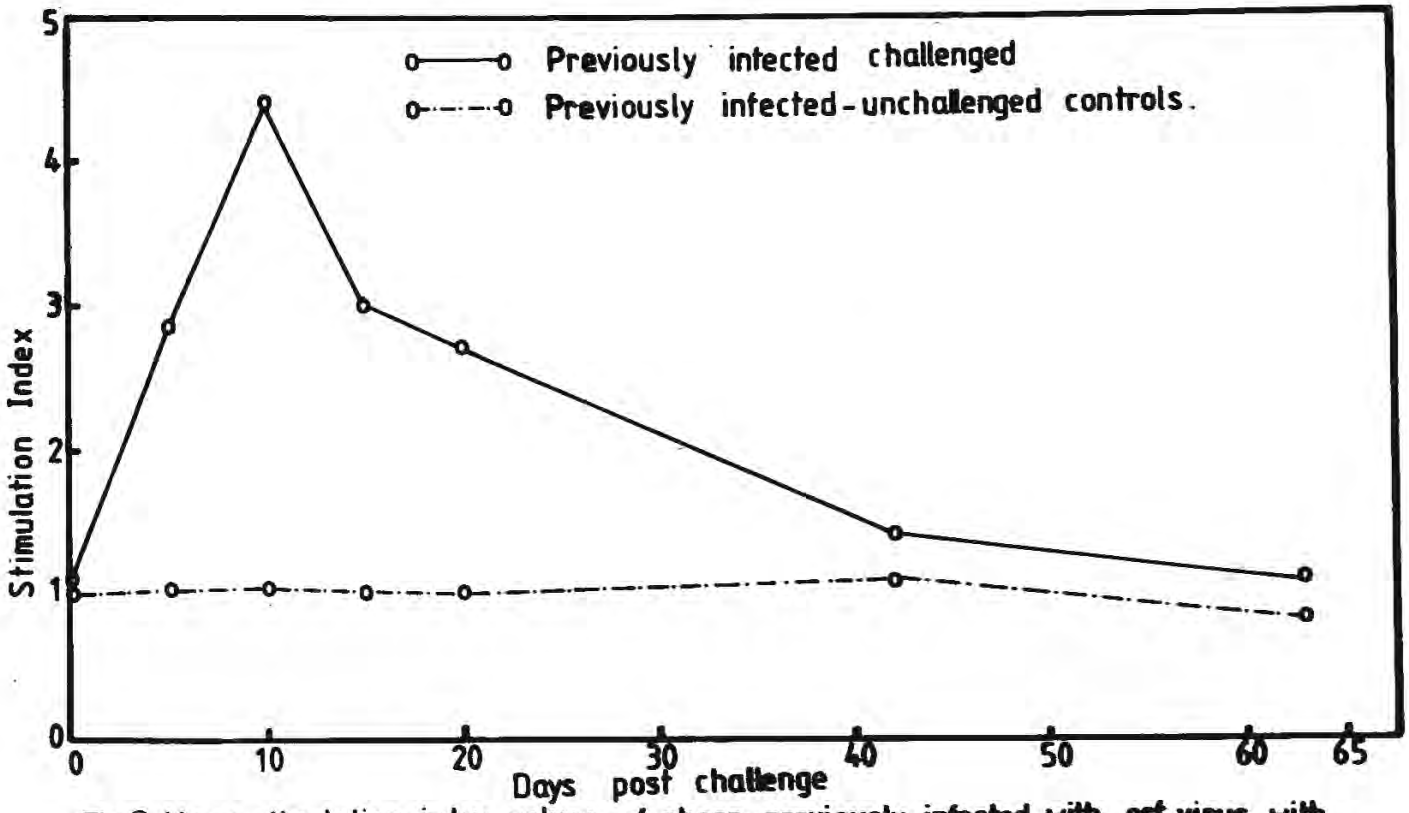

Fig 3 Mean stimulation index values of sheep previously infected with orf virus with unchallenged controls.

Figures 5: **Mean stimulation index values of sheep previously infected with orf virus with unchallenged controls**

DISCUSSION

The principal aim of this study was to establish the existence of cell-mediated immunity of orf virus in sheep, as first suggested by Aynaud (1923). The results of this study indicated that infected and re-infected sheep developed CMI as early as 4 days after infection and lasted up to 9 weeks as detected by direct LMI tests. Similarly, by LT tests, sensitized lymphocytes from re-infected sheep, were stimulated by orf antigen as shown by the high SI values, although there was no correlation between the appearance of these two parameters. All the reinfected sheep showed appreciable CMI response from the second week. The previously infected control sheep had low CMI which was significantly different from the response observed in the re-infected sheep. The preliminary migration inhibition tests, indicated that lymphokines, MIF and LIF, were produced by lymphocytes from recovered sheep when cultured in vitro in the presence of orf virus because both PE cells and PMN leucocytes had their migration inhibited. Moreover, only lymphocytes from orf-infected sheep produced LIF in the direct tests. Rockling (1974) found that sensitized lymphocyte when stimulated by specific antigen, produced distinct soluble factors. One of them was MIF which inhibited the normal guinea pig macrophages from migrating. The technique for detecting this MIF is sensitive and the results are reproducible. Another soluble factor of activated lymphocytes is the LIF which inhibited the migration of leucocytes. The data from lymphocyte transformation tests indicated that lymphocytes from sheep re-infected with orf virus were specifically stimulated by orf virus were specifically stimulated by orf antigen in vitro resulting in blast transformation thus providing evidence of CMI responses in infected sheep. The amount of radioactive thymidine uptake by the orf antigen-stimulated lymphocytes was always lower than the amount taken up by mitogen PHA-stimulated lymphocytes, but always higher than the amount taken by the non stimulated lymphocytes, Gold and Peacock (1970) reported that only 5 to 30% of lymphocytes in culture were transformed by specific antigen because only relatively small numbers of lymphocytes in the donor body became sensitized to the antigen. On the other hand, non-specific stimulation by mitogen PHA causes 50 to 70 of lymphocytes in the culture to transform because both T-cells and B-cells are being stimulated. Therefore, the results obtained in this study were in agreement with their findings.

Moreno-lopez (1977) was able to detect CMI response in calves re-vaccinated with parainfluenza 3 virus. Therefore, the results obtained in this study by showing blastogenesis of sensitized lymphocytes and migration inhibition of leucocytes strongly suggest involvement of CMI in the recovery of sheep to orf re-infection.

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