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PRODUCTION OF ANTI-*M. LEPRAE* ANTIBODIES IN MAN AND MICE UNDER THE IMPAIRMENT OF CELL MEDIATED IMMUNITY

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Immunological characteristics of lepromatous leprosy, that is specific failure of cell-mediated immunity (CMI) to *Mycobacterium leprae* in contrast to normal or elevated production of the antibodies to various antigens, suggests that humoral immune response to *M. leprae* does not necessitate the function of helper T cells and that immunological blocking may operate on the failure of CMI. In order to clarify these possibilities it is necessary to examine the both of humoral and cellular immune responses to the same antigens of *M. leprae*. Indirect fluorescent antibody technic may be suitable for such purpose, because the test is possible to detect not only classic but also blocking antibodies to *M. leprae*. Using this technic we examined the effect of immunosuppression on antibody production in the mouse infected with *M. leprae* and the relationship between the antibody titer and CMI in leprosy patients.

MATERIALS AND METHODS

1) *M. leprae* inoculation in immunosuppressed mice
A protocol of this experiment is shown in Table 1.

Table 1. Protocol of Experiment

Mouse: ddY-SLC(SPF) thymectomized at 4.5 weeks after birth

Inoculation with *M. leprae* at 5th week after thymectomy:
- FP-----left and right foot-pads (3.8×10^4 bacilli/site)
- V+FP---tail vein (1.5×10^7 bacilli) + right foot-pad (3.8×10^4 bacilli)

Immunosuppression:
- T------thymectomy only
- $T+IC_0$----thymectomy + Imuran & Cortisone from the time of inoculation
- $T+IC_{15}$---thymectomy + Imuran & Cortisone from 15 weeks after inoculation
- $T+A_0$-----thymectomy + Anti-thymocyte globulin (ATG) from the time of inoculation
- $T+A_{15}$----thymectomy + ATG from 15 weeks after inoculation

Examination: three to five mice of each group were sacrificed at intervals of 10 weeks from 20th to 70th week after inoculation.

Imuran was administered intraperitoneally with a dosage of 0.4mg to each mouse, on every days in the first week, then at intervals of 2 or 3 days in the second and subsequent weeks. Cortisone was injected intramuscularly with a dosage of 0.5mg to each mouse, at intervals of 1-3 days. Anti-thymocyte globulins (ATG) were prepared, by ammonium sulfate precipitation and DEAE-cellulose column chromatography, from a pooled serum of the rabbits which had been injected intravenously, twice at intervals of 2 weeks, with the suspension of mouse thymocyte ($2x10^8$ cells/rabbit).

2) Leprosy patients

A cooperative program, immunological studies on leprosy in Okinawa, has been initiated since 1972 and is still in progress. Both inpatients in the National Leprosaria, Okinawa Airaku-en and Miyako Nansei-en, and outpatients in the skin clinics of Naha and Hirara were surveyed and tested with lepromin. The blood taken from a part of these patients were tested with the in vitro CMI and serological reactions.

3) Immunological tests

a) In mice: Sheep red blood cells (SRBC, $4x10^8$) were injected into peritoneal cavity at 4 days before the sacrifice and the spleen cells were tested for plaque formation with SRBC in an agar plate. A part of spleen cells were also tested with in vitro transformation to PHA. The sera of mice in each group were pooled, absorbed with BCG polysaccharide, and tested with indirect immunofluorescence, using a smear of M. leprae and rabbit anti-mouse globulin fluorescent antibody. The serum of ATG-treated mouse was also absorbed with normal rabbit gamma globulin.

b) In leprosy patients: Lepromin reaction was read at 4 weeks after the intradermal injection of lepromin (160 or 40 million bacilli/ml). The size of induration, 3mm or larger, was read to be positive, according to the criteria reported previously[1]. The method of indirect fluorescent leprosy antibody absorption (FLA-ABS) test has been described in LSM[2]. Lymphocyte transformation test (LTT) was performed as follows: peripheral blood lymphocytes were purified by fractional centrifugation using Ficoll-Conray solution (sp.w. 1,085) and cultivated in a culture medium RPMI1640 containing 20% autologous serum and Dharmendra antigen ($3x10^6$ bacilli/tube). The rate of blast cell formation (stimulation index) was determined by ^3H-thymidin uptake after incubation at 37°C for 6 days.

RESULTS AND DISCUSSION

1) Immune responses of immunosuppressed mice

The effect of immunosuppressive treatment on CMI was confirmed by the inhibition of in vitro transformation of spleen cells to PHA, as shown in Table 2.

Table 2. In vitro Transformation of Spleen Cells

Group	Weeks after inoculation (1)	(5)	(15)	50	60	70	Average	%
Control	11.81	27.86	4.55	1.13	9.04	2.37	9.46	100
T		6.33	0.94	1.35	3.11	1.28	2.60	27
$T+IC_0$	2.26	10.75	2.34		3.66	2.11	4.22	45
$T+IC_{15}$				0.68	2.63	1.32	1.54	16
$T+A_0$	2.04	1.65	1.20	0.96			1.46	15
$T+A_{15}$				0.83	1.25		1.04	11

All values in the table indicate a stimulation index to PHA.

Weeks in parentheses are the period of immunosuppression to non-infected mice.

The thymectomy itself shows significant effect and more intensive suppression was achieved by thymectomy plus ATG.

It has been well known that the hemolysin production in mice is thymus-dependent, in other words it needs helper T-cells. This was also confirmed in our experiment, as shown in Table 3.

Table 3.
The number of Plaque Forming Cells to SRBC

Group	Weeks after inoculation 20	30	40	50	60	70	Average	%
Control	455	398	136	142	113	251	249	100
T	197	396	148	200	86	458	248	100
$T+IC_0$	69	455	205	307	263	86	230	92
$T+IC_{15}$	81	340	72	56	103	139	132	53
$T+A_0$	44	126		122			97	39
$T+A_{15}$	40	82	24	113	51		62	25

Although thymectomy alone shows no difference in average compared with the control group, apparent diminution of plaque-forming cells to SRBC is seen in thymectomy plus ATG group. The effect of Imuran and Cortison is not so distinct in this experiment. Some differences in suppressive effect are noted according to the starting point of the treatment, but the reason is not clear.

Table 4. Anti-M. leprae Antibody Titer by Indirect Immunofluorescence

Group		Weeks after inoculation 20	30	40	50	60
Control	FP	640	10,240	640	160	(-)
	V+FP		640	640	40	10
T	FP	640	2,560	640	640	(-)
	V+FP		640	2,560	2,560	40
$T+IC_0$	FP	2,560	640	40	640	(-)
	V+FP		2,560		640	10
$T+IC_{15}$	FP	2,560	640	40	40	10
	V+FP		2,560	640	160	(-)
$T+A_0$	FP	2,560	2,560		160	
	V+FP		640			
$T+A_{15}$	FP	640	2,560	10,240	160	10
	V+FP		2,560	2.560	640	(-)

Table 4 shows the titer of antibodies to M. leprae in indirect fluorescent antibody test. High antibody titers are seen in the group of immunosuppressed mice and there is no significant difference among the groups. However, the titers at 60th week are generally low. This may be due to the ageing of mice.

Based on these results we may consider that the production of anti-M. leprae antibodies is thymus-independent.

2) Bacteriological and histopathological findings in mice

Another purpose of this experiment was to find out the best method of immunosuppression for making lepromatous model in mice. The number of acid-fast bacilli in the foot-pad and in the other tissues increased remarkably in the thymectomized and ATG-treated mice. However, the contamination of M. lepraemurium occurred in some mice at the late stadium of experiment, so we gave up the idea of collecting complete data.

3) Correlation between humoral and cellular immune responses in leprosy patients

If the antibodies to M. leprae could blockade CMI, leprosy patients should show a reverse correlation between these two immune responses. I reported previously[3] that the lepromin test showed reverse correlation with Middlebrook-Dubos test and with Leproagglutination test, but this relationship might be only a pretense because the antigens concerning these reactions are different with each other. Supposing the same antigens cause the lepromin and FLA-ABS tests, we examined the correlation between these reactions in leprosy patients. The result is shown in Figure 1.

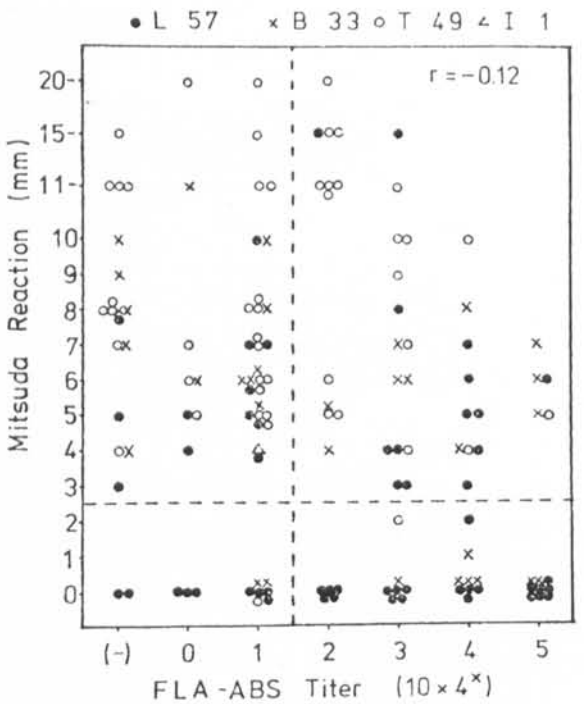

Fig.1. Correlation between lepromin and FLA-ABS tests

If a correlation coefficient (r) is calculated simply, it becomes −0.12, the value being not significant statistically. This may be due to the bimodial distribution of the readings in each test. So, a borderline was drawn at the position corresponding to the valley between two peaks and the numbers of cases divided in four areas were examined by χ^2-test. As shown in Table 5, the result is statistically significant. This means that we cannot neglect the reverse correlation between the two tests.

Table 5.
Correlation between Mitsuda reaction and FLA-ABS test

		Mitsuda		
		Neg.	Posi.	Total
FLA-ABS	Low	12	55	67
	High	30	43	73
	Total	42	98	140

$\chi^2 = 8.94$ $P < 0.01$

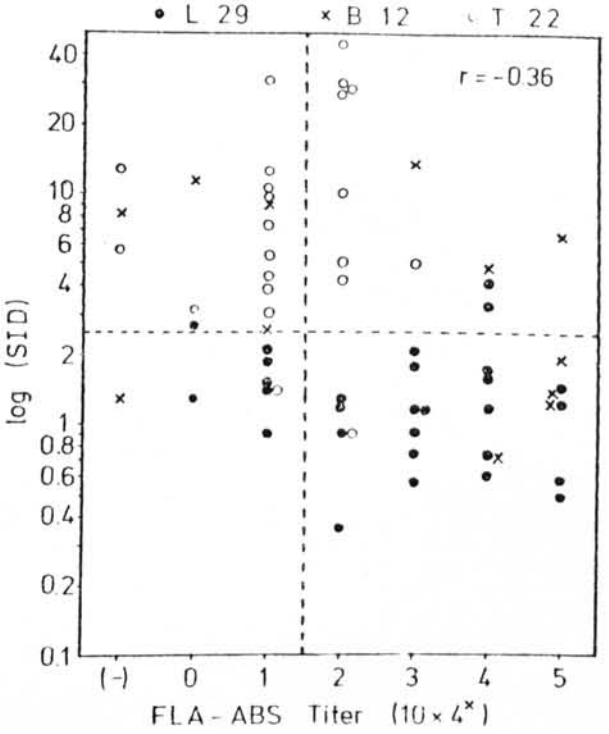

Fig.3. Correlation between LTT and FLA-ABS test

Figure 3 shows the correlation between LTT and FLA-ABS test. Although the correlation coefficient takes negative value, that means a reverse correlation, it is not significant statistically. However, as shown in Table 7, χ^2-test is significant. This fact suggests the blocking of LTT by anti-M. leprae antibodies in autologous serum. However, the number of exceptional cases which showed both low and/or high readings are not negligible. This was also true in the reverse correlation between lepromin and FLA-ABS tests. Such exceptions suggest the possibility that the antigen reacting with antibodies is not the same as that with lymphocytes because of heat-denaturation of bacilli in the latter case and therefore the reverse correlation may be only a pretense.

Table 7.
Correlation between lymphocyte transformation test (LTT) and FLA-ABS test

		LTT		
		Low	High	Total
FLA-ABS	Low	8	17	25
	High	25	13	38
	Total	33	30	63

$\chi^2 = 6.90 \quad P < 0.01$

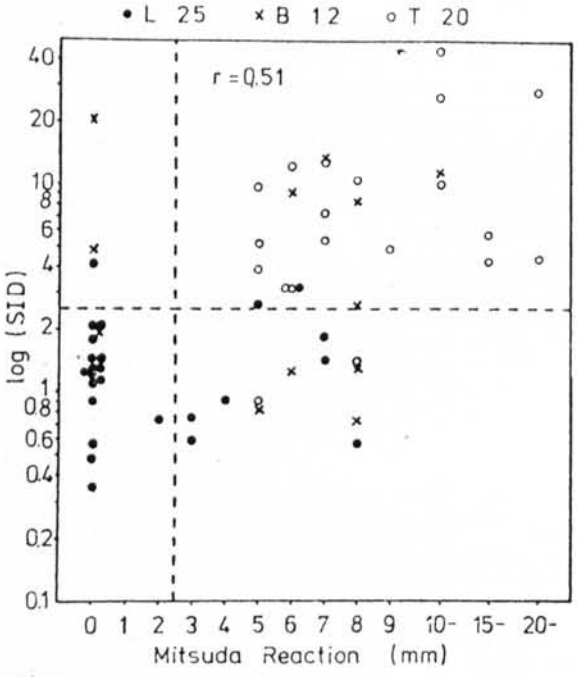

Fig.2. Correlation between lepromin test and LTT

Figure 2 shows the correlation between lepromin test and LTT. The ordinate is expressed by logarithm of SID, that is a stimulation index to Dharmendra antigen. The correlation coefficient is statistically significant. As log(SID) also shows bimodial distribution, another statistical evaluation is carried out by χ^2 test. As shown in Table 6, the correlation between these tests is apparent.

Table 6.
Correlation between lymphocyte transformation test (LTT) and Mitsuda reaction

		LTT	
		Low	High	Total
Mitsuda	Neg.	17	3	20
	Posi.	12	25	37
Total		29	28	57

$\chi^2 = 12.33$ $P < 0.001$

Alternative explanation is to connect the exceptional cases with their anamnestic or clinical findings. So, we divided the cases tested with the lepromin and FLA-ABS into four groups as shown in Table 8 and Table 9.

Table 8. IMMUNE RESPONSES AND CLINICAL FINDINGS

Mitsuda test / FLA-ABS test		Negative / Low		Positive / Low		Negative / High		Positive / High	
		No.	%	No.	%	No.	%	No.	%
Inpatient		3	25.0	28	50.9	11	36.7	18	41.9
Out-patient		9		27		19		25	
Consanguineous patient	Present	6	50.0	24	43.6	14	46.7	16	37.2
	Absent	6		31		16		27	
Anamnestic disease	Exist	3	37.5	18	36.7	3	14.3	5	14.7
	None	5		31		18		29	
Type	Lepromatous	9	75.0	12	21.8	22	73.3	14	32.6
	Borderline	2	16.7	14	25.5	7	23.3	10	23.3
	Tuberculoid	1	8.3	28	50.9	1	3.3	19	44.2
	Indeterminate			1	1.8				
Stage	Progressive	2	33.3	6	14.3	15	60.0	3	9.7
	Retrogressive	3	50.0	11	26.2	9	36.0	12	38.7
	Quiescent	1	16.7	13	31.0	1	4.0	6	19.4
	Arrested			12	28.6			10	32.3

The first and fourth groups indicate exceptional cases. The percentage of inpatient and the presence of consanguineous patient showed no significant difference among the four groups. Anamnestic disease seems to increase the number of cases which showed low FLA-ABS titer, while no effect on lepromin test. Of these cases tuberculosis and malaria were the most frequent diseases. The first group showed high percentage in the cases with lepromatous type, retrogressive stage, many skin lesions and in the bacilli-positive cases. These facts seem to indicate that both humoral and cellular immune responses could be suppressed in the advanced lepromatous leprosy. Another exceptional, the fourth group should be explained by the different way, because it does not closely related with disease type but it was few in progressive stage, much frequent in the cases with partial, macular skin lesions and also in the bacilli-negative cases.

Table 9. IMMUNE RESPONSES AND CLINICAL FINDINGS

Mitsuda test / FLA-ABS test		Negative Low		Positive Low		Negative High		Positive High	
		No.	%	No.	%	No.	%	No.	%
Skin lesion	Present	11	91.7	27	50.9	25	86.2	26	61.9
	Absent	1		26		4		16	
Leprom & infiltrat.		7	63.6	2	7.4	14	56.0	7	26.9
Macular lesion		4		25		11		19	
Number	Many	6	75.0	13	59.1	20	83.3	14	66.7
	Few	2		9		4		7	
Extent	Whole body	5	50.0	4	16.0	12	48.0	4	16.7
	Partial	5		21		13		20	
Eye lesion	Present	0		4	7.3	1	3.4	2	4.8
	Absent	11		51		28		40	
Nasal lesion	Present	2	16.7	4	7.3	1	3.3	1	2.4
	Absent	10		51		29		41	
ENL	Present	0		3	5.5	2	6.7	5	12.5
	Absent	12		52		28		35	
Other reaction	Present	0		0		2	6.7	2	5.0
	Absent	12		55		28		38	
Bacilli	Positive	5	83.3	6	14.0	16	76.2	9	32.1
	Negative	1		37		5		19	

Therefore, this means the retention of antibody production after the improvement of clinical signs due to the recovery of CMI. In other words, this may be the complete form of immune responses.

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