Early Immune Responses in Nude Mouse Following Intravenous Injection of *Mycobacterium leprae*

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(Received for Publication, August 18, 1977)

Antibody production is known to be principal function of B cell immune system. Some antigens, for example, sheep erythrocytes, induce in mouse the production of antibodies which necessitates helper function of T cells. Such antigen is called thymus-dependent¹⁾. The antigen of *M. leprae* seems to be thymus-independent, because the antibody-production to this antigen has been demonstrated, as reported previously²⁾, in the patients with lepromatous leprosy as well as in immunosuppressed mice both of which are considered to be under the impairment of cell-mediated immunity (CMI). However, there is no evidence that the function of helper T cells is also impaired. In order to elucidate this problem, athymic nude mouse may be useful, because the animal is known to be lacking in T cell functions while normal in B cell system³⁾. Such an unbalance in immune system may permit an enormous multiplication of *M. leprae*, as successfully demonstrated by Kohsaka et al⁴⁾.

Another purpose of present study is to clarify immune responses in nude mice at early stage after the infection of *M. leprae*. Such study is necessary for evaluating immunological tests for leprosy, i. e. indirect fluorescent antibody test and lymphocyte transformation test, both of which have been developed recently and are expected to be useful for early diagnosis of leprosy.

Materials and Methods

1) Animals

Specific pathogen-free, BALB/c-nu/nu (homozygote, 6 female) and BALB/c-nu/+ (heterozygote, 6 male) mice were purchased from Central Laboratory for Experimental Animals (CLEA) Japan Inc., and conventional BALB/c-+/+ (2 male and 5 female) mice were offered from the laboratory of epidemiology in author's institute. These mice were maintained in the metal cage with filter-cap and fed with sterilized diet and water.

2) Administration of antigens

A suspension of *M. leprae* was prepared from lepromatous nodule which had been removed from relapsed patient and kept in a deep-freezer at -20°C for nearly two months. One tenth ml of saline suspension consisted of 10 million bacilli and 100 million sheep red blood cells was injected intravenously to each mouse at 5-7 weeks age. Six nude mice were reinjected with the same antigens at 5th week after the initial injection.

3) Transfer of thymocytes

Six nu/+ mice were sacrificed to obtain thymus cells at 4th week after the injection of antigens. Thymus cells were pooled in saline and passed through a glass-fiber column. The harvests, 90 million cells were divided into three equal parts and injected intravenously to three out of six nude mice.

4) Serological tests

The blood was taken from tail vein of each mouse using capillary tubes, on the fixed days of the experiment. The method of indirect fluorescent antibody test has been described elsewhere^{2,5}. The smears of *M. leprae* were prepared from subcutaneous leproma of armadillo (L-154 (456)) which had been offered from Dr. E. E. Storrs, Gulf South Research Institute, New Iberia, Louisiana, U. S. A. Instead of fluorescent antibody to human gamma globulin, the following two reagents were used as second antibody: Rabbit antiserum to mouse serum was prepared in author's laboratory and labelled with fluorescein isothiocyanate by dialysis method⁶, and goat fluorescent antibody to mouse IgG was purchased from Hyland Laboratories, Los Angeles, California, U. S. A. The antibody-titer was expressed by the maximum dilution of mouse serum showing 2+ fluorescence of bacilli in the light passed through BV or interference filter system. Agglutination and hemolysin titers to sheep red blood cells were determined with microtiter plates.

5) *In vitro* test on transformation of spleen cells

This test was performed at the sacrifice of mice. Ten to 30 million spleen cells/tube were mixed with sonicated suspension of *M. leprae* in the medium RPMI 1640 containing 5% human serum and cultured at 37°C for 4 to 5 days in a CO₂-incubator. The transformation of stimulated cells was measured by the uptake of tritiated thymidin which was added to each tube prior 24 hours of the end of cultivation. The stimulation index (SI) was expressed by the ratio of average radioactivity (cpm) in triplicate test tubes containing antigen to that in controls without antigen.

Results and Discussion

Macroscopically, all mice showed no external nor internal lesion, except the lack of hair and thymus in nu/nu mice. Anti-*M. leprae* antibody-titer curves are shown in Fig. 1. The shape of curves in each group of mouse indicated clear difference according to the use of different fluorescent antibodies up to two weeks after the first antigen-injection. This may be due to initial response of IgM antibodies followed by delayed production of IgG antibodies. It is noteworthy that antibody-titer reaches the maximum within 1 to 2 weeks after the injection of *M. leprae*. This seems to support the usefulness of indirect fluorescent antibody technique for early serodiagnosis of leprosy. The antibody-titer in nu/nu mice were not significantly different from those in the other two groups. This fact indicates that antibody production to *M. leprae* is thymus-independent. The transfusion of thymocytes from nu/+ donor did not influence the antibody-titer of recipient nude mice, as shown in the upper part of Fig. 1. Second injection of antigens tends to increase the antibody-titer irrespective of transferring thymocytes (solid line) or not (broken line). As these mice had

Fig. 1 Anti-*M. leprae* antibody titers of mouse serum determined by rabbit fluorescent antibody to mouse serum ($-O$, $\bullet-$) and goat fluorescent antibody to mouse IgG ($-\Delta$, $\blacktriangle-$)

Fig. 2 Antibody-titers of mouse serum in agglutination ($-O$, $\bullet-$) and hemolysis ($-\Delta$, $\blacktriangle-$) reactions with sheep red blood cells

to be sacrificed for examining the transformation of spleen cells, further course of secondary immune response could not be followed up.

The antibody-response to sheep red blood cells is shown in Fig. 2. This antigen is known to be thymus-dependent¹¹. In fact, the production of agglutinins and hemolysins in nude mice were significantly lower than those in the other mice possessing thymus. As shown in the middle part of Fig. 2, nu/+ mice maintained enough high antibody-titer by the time of sacrifice. However, the thymocyte-transfer alone did not induce antibody-production in nude mice when examined at 6th day after the transfer. Both agglutinins and hemolysins appeared in T cell recipients at 4th day after the second injection of antigens. However, the same titer of hemolysin was also observed in non-recipients. Then, the effects of 2-mercapto-ethanol (ME) on these antibodies was tested with the serum from sacrificed mouse. As shown in Table 1, ME resistant antibody was produced by the most of control mice, but never by any of nude mice, irrespective of the thymocyte-transfer and even after the second injection of antigens. It might be necessary to observe further course of secondary immune response for demonstrating helper function of transferred thymocytes.

In vitro transformation of mouse spleen cells stimulated by *M. leprae* antigens is shown in Table 2. This reaction is considered to be an index of cell-mediated immunity (CMI). When the animal was sacrificed within 6 weeks, no BALB/c nor nu/+ mouse caused positive re-

Table 1 The effect of 2-mercapto-ethanol (ME) on the antibody-titer to sheep red blood cells

Mouse	No. of animal	Agglutinin titer*		Hemolysin tier*	
		no ME	ME treated	no ME	ME treated
nu/nu+thymus cells	1	2	(—)	3	(—)
	2	1	(—)	1	(—)
	3	3	(—)	2	(—)
nu/nu no thymus cells	4	0	(—)	2	(—)
	5	0	(—)	1	(—)
	6	2	(—)	4	(—)
nu/+	7	3	3	2	1
	8	4	3	4	1
	9	5	3	3	1
	10	3	3	2	1
	11	4	3	1	0
	12	4	(—)	2	0
BALB/c	13	7	5	5	5

* The titer is expressed by x in 10×2^x .

Table 2 *In vitro* transformation of mouse spleen cells to *Mycobacterium leprae* antigens

Mouse	No. of animal	Week of sacrifice	no antigen cpm	³ H-thymidin uptake of cells with			
				2.5×10 ⁶ bacilli cpm	S.I.*	5×10 ⁶ bacilli cpm	S.I.*
nu/nu+thymus cells	1	5.5	1517	—	—	1252	0.83
	2	5.5	3642	—	—	3632	1.00
	3	5.5	1227	—	—	1745	1.42
nu/nu no thymus cells	4	5.5	3003	—	—	2402	0.80
	5	5.5	3021	—	—	1833	0.61
	6	5.5	1886	—	—	1698	0.90
nu/+	10	4	1239	1205	0.97	693	0.56
	11	4	210	139	0.66	134	0.64
	12	4	362	356	0.92	410	1.13
Conventional BALB/c	13	4	4177	—	—	4594	1.10
	14	16	357	—	—	682	1.91
	16	16	811	—	—	867	1.07
	17	16	791	—	—	907	1.15

* S.I.=stimulation index

sponse which was shown by the stimulation index larger than 1. However, only one nude mouse (No. 3) caused positive response after the transfusion of thymus cells from nu/+ donors followed by the second injection of antigens, while the other nude mice receiving no thy-

mocyte remained negative. Among three conventional BALB/c mice sacrificed at 16th week, one mouse (No. 14) also showed positive response. Therefore, if the thymocyte-transfer and the test with spleen cells would be performed at far late stage, more intense response in the recipient mice might be observed. Such condition must be considered in future study.

It is generally known that CMI to intracellular parasite is actively induced when the micro-organisms are able to multiply to some extent in the host cells⁷⁾. Since the presence of viable bacilli in the material could not be confirmed, relatively large number of *M. leprae* was injected intravenously. In such condition, a large quantity of antigens liberated from dead bacilli may activate B-cell system and lead to early production of antibodies without inducing CMI. Even if competent T cells are activated, but if insufficient in their number, large quantity of antigens may deplete the function of T cell system. Such mechanism, i. e., immunoparalysis, may operate in these mice at early immune response to *M. leprae* infection, and this may be a model of indeterminate leprosy having a tendency to lepromatous type.

On the other hand, if small number of viable *M. leprae* are infected locally, they are ingested in phagocytes, and processed antigens or direct cell-cell contact may activate T cell system without inducing antibody response. This may be another course of indeterminate leprosy having a tendency to tuberculoid type. If similar processes are realized in nude mouse by passive transfer of CMI, it will provide us very useful model for the studies on prophylaxis and therapy of lepromatous leprosy.

Summary

For the purpose of elucidating immune mechanism in early stage of leprosy, three strains of mice, conventional BALB/c, SPF BALB/c-nu/+ and BALB/c-nu/nude mice, were injected intravenously with a mixture of 10^7 *M. leprae* and 10^8 sheep red blood cells. All of these mice showed similar degree of antibody response to *M. leprae*, as demonstrated by indirect immunofluorescence, the antibody-titer reaching to the maximum within a week after the injection of antigens. The production of IgG antibodies was somewhat delayed and the titer reached to a plateau within 2 or 3 weeks. No decline of antibody-titer was observed till at least 5 weeks after the injection of antigens. The transfer of thymocytes from immunized nu/+ donors to nu/nude recipients did not influence the antibody-titer in the recipient.

Both agglutinins and hemolysins to sheep red blood cells were less produced in nu/nude mice than in nu/+ and conventional mice. After the transfer of thymocytes followed by second injection of antigens, nude mice showed low level of antibody response which was sensitive to mercapto-ethanol treatment. *In vitro* transformation of spleen cells to *M. leprae* antigens was not caused by any of mice, except for positive response by one nude mouse into which thymocytes from immunized donor and second injection of antigens were given.

From these observations it may be concluded that the production of anti-*M. leprae* antibodies is thymus-independent and that this immune response can be induced at early stage of leprosy infection without induction of cell-mediated immunity.

Acknowledgement

This investigation was partially supported by the grant of US-Japan Cooperative Medical Science Program and that of Ohyama Health Foundation.

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らい菌の静脈注射後に惹起されるヌードマウスの 早期免疫応答

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(受付: 1977 年 8 月 18 日)

らいの初期免疫機構を明らかにするために、通常の BALB/c, SPF の BALB/c-nu/+ 及び BALB/c-nu/n nu の 3 種類のマウスに、らい菌 10^7 コとヒツジ赤血球 10^8 コの混合液を静脈注射した。間接免疫蛍光法でしらべた抗らい菌抗体応答はすべてのマウスで同程度に起り、抗体価は抗原注射後 1 週以内に最高に達した。IgG 抗体の産生はややおくれ、抗体価は 2~3 週以内に水平位に達した。抗原注射後少くとも 5 週以内には抗体価の減少は認められなかった。免疫した nu/+ マウスの胸腺細胞を nu/n nu マウスに移入したが、後者の抗体価に影響を及ぼさなかった。これらの所見はらい菌に対する抗体産生が胸腺非依存性であり、また間接蛍光抗体法が感染直後の抗体産生を見いだすのに役立つことを示している。

ヒツジ赤血球に対する凝集素及び溶血素の産生は通常の BALB/c 及び nu/+ マウスよりも nu/n nu マウスでは弱く、この免疫応答が胸腺依存性であることを示した。胸腺細胞を移入し、ついで再度抗原注射を行ったが、nu/n nu マウスの抗体価は低く、抗体は 2-メルカプトエタノール処理により失活した。

らい菌抗原に対する脾細胞のガラス器内変形反応は、胸腺細胞を移入し、抗原再注射を行ったヌードマウスの 1 匹で陽性となったほかは、どのマウスでも陰性であった。これは大量のらい菌抗原の静脈注射が T 細胞の枯渇を起した故かも知れない。このような、細胞性免疫の誘導なしに液性免疫応答が感染初期に起ることは、らい腫型に傾き易い未定型らいのモデルをあらわすように思われる。