Acta Medica Okayama

Volume 31, *Issue* 6 1977 *Article* 1

DECEMBER 1977

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Yukio Hamazaki*

*Okayama University,

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*PMID: 147604 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **31**, 343—349 (1977)

THE IMMUNOLOGICAL RELATIONSHIP BETWEEN FILTRABLE AGENT, *SALMONELLA* AND MURINE LEUKOSIS

Yukio HAMAZAKI

Department of Pathology, Okayama University Medical School, Okayama 700, Japan

Received August 24, 1977

Abstract. *Salmonella typhimurium* was invariably isolated from our J strain murine leukosis. Immunization of D103 mice with either inactivated *Salmonella typhimurium* or the cell-free extract of leukosis inhibited the transplantation of leukosis. The adoptive immunization of D103 mice with spleen cells of Strong A mice immunized with either *Salmonella* or the cell-free extract of leukosis inhibited the transplantation of leukosis. The addition of either *Salmonella* or the cell-free extract of leukosis inhibited the migration of macrophages of leukosis spleen in tissue culture. Strong A mice is non-susceptible to J strain leukosis. However, inoculation of neonatal Strong A mice with the cell-free extract of leukosis produced a susceptiblity to the transplantation of leukosis. These results suggest that both a filtrable agent and *Salmonella typhimurium* are present in cells of this leukosis and might be etiologically related to the leukosis.

The author has conducted long term studies on feeding young rats and mice on fresh human malignant tumor tissues obtained during surgery or autopsy. Since 1963 such experiments have been performed 85 times, and leukosis has developed 28 times (33%). In 18 of these strains, serial transplantations were attempted. The cell-free extact induced leukosis when injected into mice within 24 hours of birth but not in adult mice (1-4). Many inbred mice were susceptible to this leukosis. The D103 mice were most susceptible, and the Strong A mice were not susceptible at all. This cell-free extract was rather stable and remained active for about 2 years if the spleen or liver tissue was immersed in 50% glycerin and kept at $-30°$C. The leukosis was myelogenous and produced moderate enlargement of the spleen and liver, without any remarkable changes in peripheral blood. The term "leukosis" was used, as it more accurately describes the actual condition, than the term leukemia.

It was of special interest that many *Salmonella typhimurium* bacilli were invariable and purely isolated from the first passage from the spleen or liver of mice of the J strain of our leukosis.

What implication does *Salmonella* have in this J strain leukosis? This paper reports on immunological studies on the relationships between the virus, *Salmo-*

nella bacilli and this leukosis.

MATERIALS AND METHODS

Mice. D103 and Strong A mice obtained from the mouse colony of Okayama University Medical School were used.

Leukosis. J strain murine leukosis transplanted in D103 mice was used. The history of this leukosis strain has been reported elsewhere (1-4).

Antigens. Salmonella typhimurium isolated from the spleen and liver of D103 mice afflicted with leukosis was used, and a suspension containing about 1,400 bacilli in 1 cmm of physiological saline solution was prepared. This was inactivated by mixing with formalin to 0.5% at 0°C for 24 hr. The cell-free leukosis extract was prepared by adding a five-fold volume of physiological saline solution to the spleen and liver of leukosis mice; thoroughly homogenized at 0°C; and passed through HAWP millipore filter to produce a cell- and bacteria-free extract. Control antigen was similarly prepared with the spleen and liver of normal D103 mice.

Leukosis cell suspension for challange. Leukosis spleen and liver were minced and suspended in physiological saline to 10%, and 0.03 ml was intraperitoneally injected as a challenge.

Immunization. The antigen solutions were mixed with an equivolume of Freund's incomplete adjuvant (Difco). A half or one milliliter of antigen was injected into the extremities of mice at four different sites. The injections were repeated 5-6 times at intervals of 5 days, and a month later the challenge suspension was injected.

Adoptive immunization test. Salmonella bacilli antigen (0.1-0.15 ml) or the cell-free extract of the leukosis spleen and liver (0.5-1.0 ml) was intraperitoneally injected into Strong A mice, which are non-susceptible to this leukosis. This was repeated three times at intervals of 5 days, and 5-7 days after the last injection, the mice were bled to death. Spleen lymphatic cells removed from these animals were intraperitoneally injected into recipient D103 mice. The number of cells injected into each animal was about 5×10^7. At 5 to 10 days after cell transplantation, the challenge leukosis cell suspension was intraperitoneally injected.

In the control group, a similar number of spleen cells of non-treated, normal Strong A mice were transplanted into D103 mice, and similarly challenged.

Macrophage migration inhibition test. Leukosis spleen cells were intraperitoneally injected into D103 mice, and when the splenomegaly palpated, mice were bled to death. The spleen was removed, and tissue specimens of about 2 mm^2 were obtained from the parenchyma. These tissue specimens were fixed to the bottom of small dishes about 4 cm in diameter with plasma obtained from Strong A mice, and maintained in Eagle's minimum essential medium supplemented with 20% calf serum. In the experimental group three antigens were added to the culture medium: (a) suspension of *Salmonella typhimurium* isolated from leukosis, (b) cell-free extract of the spleen and liver of leukosis,

and (c) a mixture of these two antigens. The optimum antigen amount was 0.2ml per 5ml of culture medium. After culture for 24 hr at 37°C in a 5% CO_2 incubator, the culture medium was removed, floating cells (mostly lymphocytes and leukocytes) were washed off, and cells fixed to the glass surface (mostly macrophages) were rapidly dried by a fan, fixed and stained by Giemsa stain. The migration distance was measured under a microscope with an ocular micrometer. Measurements were performed in six spleen specimens, and the average migration distance was computed.

Immunological tolerance test. Newborn Strong A mice non-susceptible to leukosis were used as test animals. As pretreatment 0.1-0.15ml of the cell-free leukosis extract was intraperitoneally injected into newborns. At 35 to 40 days after pretreatment, mice were given intraperitoneal injections of the leukosis cell suspension.

RESULTS

Immunization with Salmonella and cell-free extract

D103 mice immunized with *Salmonella* from leukosis and challenged with the leukosis cell suspension showed an average survival period of 49 days while control animals showed a survival period of 16 days (Table 1).

TABLE 1. IMMUNIZATION WITH *Salmonella* AND CELL-FREE EXTRACT

Antigen used for immunization	No. of mice[a]	Days from leukosis cell transplantation to leukosis death	
		Range	Mean
Salmonella from leukosis[b]	6	20- 66	49
None	5	9- 12	16
Cell-free extract of leukosis spleen and liver	6	39-112	61
Cell-free extract of normal spleen and liver	6	5- 11	8

a D103 mice immunized with antigen and challenged a month later with leukosis cell suspension.

b Inactivated with formalin.

When the cell-free extract of leukosis was injected and challenged with the cell suspension of leukosis, the average survival period was 61 days, and it was 8 days in the control group (Table 1).

Adoptive immunity

A general principle in adoptive immunity is that live cells be used as antigen (5). *Salmonella* may thus be antigen in adoptive immunity, but viruses are not cells in this sense. A rule in virus use as antigen in adoptive immunity is that the sensitive animals are first infected with the virus, and the virus-infected cells are used as antigen in adoptive immunity (6). However, our murine leukoses were invariably infected with *Salmonella*, and virus-infected cells, void of *Salmonella*

cannot be collected (7, 8). Therefore, the cell-free leukosis extract was used as the antigen.

Antigen from live *Salmonella* cultured on agar slopes for 24 hr produced an extended life span in the experimental group (Table 2, Experiment I).

On the other hand, when inactivated *Salmonella* was used as antigen, no difference in survival was observed between the experimental and control mice (Table 2, Experiment II and III).

When the cell-free extract of leukosis was used as antigen, the life span of mice of the experimental group was significantly extended in comparison with that of control animals (Table 2, Experiment IV). When the cell-free extract was heated to 56° C for 30 min, no effect was observed (Table 2, Experiment V).

Macrophage migration inhibition

In specimens cultured in the absence of antigens, macrophage migration was very clearly observed (Fig. 1a). Cells lying parallel extended widely and displaced the substrate (coagulated plasma) on the glass surface to form a coating in the periphery. On the contrary, in specimens cultured in the presence of antigens, migrating cells aggregated irregularly about the tissues, and the migration distance was short (Fig. 1b).

The migration index was calculated according to the following formula, in which MI means the migratory index, and MD_1 and MD_2 are the mean values of the migration distances in the control and antigen-added specimens respectively.

$$MI = \frac{MD_2}{MD_1} \times 100$$

When *Salmonella* bacilli were used as antigen, MI was 33%; when the cell-free extract of leukosis was used as the antigen, MI was 32%; and when a mixture of these two antigens was used as the antigen, MI was 21% (9).

Immunological tolerance

Strain Strong A is non-susceptible to our leukosis. Of 39 Strong A mice inoculated at birth with the cell-free leukosis extract, none developed leukosis (Table 3, Control A). Another group of 30 Strong A mice receiving no treatment at birth and inoculated with the leukosis cell suspension at 35–49 days of age also did not develop leukosis (Table 3, Control B). However, in 39 mice inoculated with the cell-free leukosis extract at birth and again inoculated with the leukosis cell suspension 35–49 days later, 18 (46%) developed leukosis (Table 3, Experimental group). In comparison with leukosis in D103 mice, the liver and spleen enlargement in leukosis-developing Strong A mice was not remarkable, and the histological changes were mild. This was especially clear in mice developing leukosis after a short latent period of several weeks.

TABLE 2. ADOPTIVE IMMUNITY

No. of experiment	Group	Antigen used for immunization of donors	No. of recipient mice	No. of mice developing leukosis	Days from leukosis cell transplantation to leukosis death	
					Range	Mean
I	Experimental[a]	Live *Salmonella*[b]	5	5	42–115	63
	Control[c]		5	5	5– 10	7
II	Experimental[a]	Formalin-treated *Salmonella*[b]	5	5	6– 16	10
	Control[c]		5	5	7– 16	12
III	Experimental[a]	Heated *Salmonella*[b, d]	5	5	5– 13	9
	Control[c]		5	5	6– 10	8
IV	Experimental[a]	Cell-free extract of leukosis[b]	5	5	12– 27	17
	Control[c]		5	5	6– 12	9
V	Experimental[a]	Heated cell-free extract of leukosis[b, d]	5	5	4– 10	7
	Control[c]		5	5	4– 14	7

a D103 mice received spleen cells of Strong A mice immunized with antigens, and 5–10 days later, received a challenge of leukosis cell suspension.
b Isolated or made from the spleen and liver of leukosis mice.
c D103 mice received spleen cells of normal Strong A mice and were treated in the same way as experimental mice.
d Heat-treatment was conducted at 56°C for 30 min.

TABLE 3. INDUCTION OF IMMUNOLOGICAL TOLERANCE BY CELL-FREE EXTRACT

Group	No. of mice[a]	Pretreatment at birth[b]	Challenge by leukosis[c]	No. of mice developing leukosis (%)	Days from leukosis cell transplantation to leukosis death	
					Range	Mean
Experimental	39	Done	Done	18 (46)	32–311	160
Control A	20	Done	Not done	0 (0)		
Control B	20	Not done	Done	0 (0)		

a Strong A mice non-susceptible to this leukosis were used.
b Intraperitoneal inoculation of cell-free leukosis extract within 24 hours after birth.
c Intraperitoneal inoculation of leukosis cell suspension at 35–49 days of age.

Fig. 1. Inhibition of macrophage migration. (a), Control in which *Salmonella typhimurium* was not added to the culture-medium. May-Giemsa. ×40. (b), Spleen tissue fragment of J stain murine leukosis cultured in the presence of *Salmonella typhimurium* isolated from the same strain of leukosis. May-Giemsa. ×40.

DISCUSSION

The most remarkable characteristic of our J strain leukosis was the invariable isolation of *Salmonella typhimurium* from leukosis tissues. In leukosis induced by inoculating cell-free extract into newborn D103 mice, a large amount of *Salmonella* bacilli was invariably isolated from the spleen and liver in the first passage. This suggests that for the development of J strain leukosis, the synergetic action of specific *Salmonella* bacilli and a filtrable agent, probably a virus, is necessary.

In immunization test with D103 mice, treatment with either *Salmonella* or the cell-free extract of J strain leukosis inhibited the transplantation of leukosis. In adoptive immunity studies, transplantation of spleen cells of Strong A mice immunized with either live *Salmonella* or the cell-free extract significantly inhibited leukosis cells in mice. In macrophage migration inhibition studies, the addition of *Salmonella* bacilli or the cell-free extract remarkably inhibited macrophage migration and when these two factors were simultaneously added to the culture medium, a more remarkable inhibitory effect was observed. These results suggests that both a filtrable agent and *Salmonella typhimurium* exist in and are etiologically related in our J strain leukosis. An immunologic tolerance to leukosis transplantation was successfully induced in Strong A mice by inoculating the cell-free extract of leukosis at birth. This also suggests the presence of a filtrable agent in cells of our J strain leukosis.

The existence of leukemia virus in leukemia cells is well known in mice (10). *Salmonella* bacilli unlike common bacilli have the property of proliferating as L type bacteria in cells they occupy (facultative intracellular parasitic bacteria). For this reason they might make contact with a filtrable agent within the cell and by synergetic action with it, disturb the nucleic acid metabolism of host cells (11, 12).

Acknowledgment. The author wishes to express his appreciation to Prof. Y. Yabe, Prof. Y. Kanemasa and Dr. K. Orita, Lecturer, for their kind help and discussions.

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