Effect of Ionizing Radiation on Tumor Immunity
II. Roles of the spleen and the lymphnode cells

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腫瘍免疫に対する電離放射線の影響
第2報 脾およびリンパ節細胞の役割について

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抗体産生系については，異説のあるところであるが，抗体産生と密接な関係にある臓器が，リンパ節，脾臓，骨髄，胸腺などであることについては異論はないであろう．ことに，移植免疫においては，液性抗体の関与が乏しく，小リンパ球に関連した細胞性抗体の意義が重要視されている．

吉田肉腫に対して抗腫瘍性を獲得したRatsが，500Rの全身照射をうけることによつて，抗腫瘍性の消失が認められたことは前報において報告した．今回は，抗体産生系と考えられている種々の細胞の放射線感受性に基いて検討を行ない以下のような結果をえた．

脾を含む下半身500Rの照射をうけた免疫Ratは抗腫瘍性を消失するが，脾が保護されて全身照射をうけた場合には，抗腫瘍性は残存する．しかし，脾のみが照射されても抗腫瘍性の消失はみられない．

移植免疫において主役を演ずるものは，リンパ細網細胞系であり，500Rの照射をうけると，大部分の細胞が障害をうける．完全に回復するためには数週を要する．一方，成熟したプラズマ細胞および腹腔単核細胞（大喰細胞）は放射線に対して比較的抵抗性で，500Rの照射をうけても著明な変化はみられない．

免疫Ratsのリンパ節細胞および脾細胞は，試験管内で接触した吉田肉腫細胞の増殖能を低下させるが，500Rの照射をうけると，細胞の障害と同時に抗腫瘍性の消失もみられる．即ち，リンパ節細胞および脾細胞は，細胞性抗体の担手であると考えられる．

A piece of tumor, transplanted to a different kind of animal is usually shed off, but when this animal is preliminarily irradiated with sublethal dosis, the piece is fixed, or takes[1)2)]. In this case, it is believed, the antibody production system is damaged by the irradiation, and subsequently immunological reaction to the heterotransplantation is inhibited. According to Amano and Hanaoka[3)4)], the antibody production system is dual, consisting of lymphogonia of the lympho-reticulocyte system and plasmocytes, which were claimed by them to be the origin of pericytes of the blood vesseles. Against this, Nossal et al[5)] takes a view of monogenesis, considering that plasmocytes are also derived from the lymphocyte system. In this way, there is, genetically, divergence of opinion concerning the antibody production system. But

no one will disagree in that it is cells of both the lymphocyte and the plasmocyte system which play the principal roles in the production of antibody. We may therefore conclude that the lymph node, the spleen, the bone marrow and the thymus occupay important positions in the antibody production system. Especially in transplantation immunity, participation of humoral antibody is considered insignificant, while great importance is placed on cellular antibody related with small lymphocytes[6)7)8)].

In the previous paper[9)] we reported that when Yoshida sarcoma cells were transplanted to mice on 2 days after whole body irradiation with 500 R, the hosts failed to reject them, all dying of the tumor; and that Wistar rats which acquired transplantation immunity against Yoshida sarcoma, likewise lost it on 2 days or later following 500 R whole body irradiation. We found out, however, that when the tumor cells were retransplanted on 9 days or later following the irradiation, some of the hosts were cured despite transient proliferation of tumor cells.

In the present work we investigated the roles of the spleen and the lymphnode cells in anti-tumor immunity. And as the result we reconfirmed that antibody producing cells which play the principal part in anti-tumor immunity belong to the lympho-reticulocyte system which is extensively distributed in the spleen and lymphnodes, and that anti-tumor immunity in immunized rats is abolished by irradiation because it damages the lympho-reticulocyte system. Also the antitumor action, born by lymphoid cells in immune rats, was found to be abolished when these cells were irradiated.

Materials

Male Wistar rats were used.

Yoshida sarcoma cells were supplied by Sasaki Laboratory and transplanted through generations in this department.

In immune rats, the acquired immunity was so strong as to reject completely the challenge intraperitoneally with 10^7 Yoshida sarcoma cells. The method of immunization was the same as described in detail in the previous report[9)].

Irradiation: With the Toshiba's KXC-18 type. 150 Kv, 25 mA, 0.5 Cu + 0.5 Al, 30 cm, 0.83 mm Cu, 114 R/min., 10 x 10 cm^2.

Experimental

1. Partial irradiation of immune rats

Experiment 1.

The body of the immune rats was divided into 2 parts with the border in the ensiform process—the upper half, containing the thymus, and the lower half, containing the spleen. Irradiation was made with 500 R to one part of the body, with the other part covered with lead. At 48 hours after the irradiation, the animals were challenged by intraperitoneal transplantation of 10^7 Yoshida sarcoma cells, and ascitic fluid was taken from time to time to observe change in the immunity.

Out of 6 rats, irradiated on the lower half of the body, 4 died of the tumor after the challenge, indicating the diminution or loss of the immunity. In the dead 4 cases, the tumor cells were markedly proliferated as compared with the early stage. The death occurred between 10 and 18 days after the challenge. An especially prominent finding at autopsy was diminution of the spleen, which weighed below 0.3 g in all the 4 dead cases. When non-treated controls were challenged by the tumor inoculation, all died of the tumor; but the spleen weighed between 1 and 2 g at autopsy, indicating enlargement as compared with the normal spleen, which weighs between 0.5 and 0.7 g. In the surviving 2 cases, reactive

cells were visible immediately after the transplantation, and the tumor cells were rejected without showing proliferation. They were sacrificed on 21 days after the transplantation, and neither tumefaction nor deposition of ascitic fluid was disclosed by autopsy. The spleens weighed 0.8 and 1.2 g, respectively, which were not too small, at least not below the normal, for the rat of 220 g in body weight.

When the upper half of the body was irradiated, all except 1, that is, 5 cases still preserved the immunity strong enough to reject perfectly Yoshida sarcoma cells, which were transplanted in an amout of 10^7, indicating the maintenance of the immunity (Table 1). The one case died on 13 days after the challenge.

Table 1 PARTIAL X-IRRADIATION ON THE IMMUNIZED RATS I

IRRADIATED PART	NO. OF ANIMALS	NO. OF DEATH
THE UPPER HALF OF THE BODY	6	1
THE LOWER HALF OF THE BODY	6	4
NON-IRRADIATED IMMUNE RATS	6	0
UNTREATED CONTROL	6	6

Summary

It was reported in the previous paper that transplantation immunity, possessed by immunized rats was abolished by whole body irradiation with 500 R. When, however only the lower half body was exposed to radiation in the present experiment, 2 out of 6 cases showed the maintenance of the immunity, though it was lost in the other 4. It has significant implication that in the surviving 2 cases, the spleen was far heavier than that in the dead 4 cases, indicating a connection between the immunity and the spleen.

When the upper half body was irradiated, one died of the tumor after the challenge, but the other 5 did not display any findings indicative of the irradiations effect. This seems to show that on the immunity which has already been acquired in mature rats, the thymus has no significant effect.

Experiment 2.

The lower half body was further divided into the right and left part by the median line, and each was separately irradiated with 500 R to examine for effect of the irradiation on the transplantation immunity as in Experiment 1. But against the anticipation that the immunity will be suppressed by destruction of the spleen in the left side irradiation, and by that of the lymph apparatus in the ileocecal region in right side irradiation, the immunity remained strong enough in both cases to reject the challenge by intraperitoneal transplantation of 10^7 Yoshida sarcoma cells (Table 2).

Table 2 PARTIAL X-IRRADIATION ON THE IMMUNIZED RATS II

IRRADIATED PART	NO. OF ANIMALS	NO. OF DEATH
THE RIGHT PART OF THE LOWER HALF BODY	4	0
THE LEFT PART OF THE LOWER HALF BODY	4	0
NON-IRRADIATED IMMUNE RATS	4	0
UNTREATED CONTROL	4	4

Summary

The results seem to indicate that neither the antibody production system is lodged only in one organ, say the spleen, nor is there any organ which totally governs antibody production.

Experiment 3.

Experiment 1 demonstrated intimate relation between the transplantation immunity and the spleen. But experiment 2 disclosed that the immunity was not abolished by irradiation of the left lower half of the body, which contains the spleen. Now, in order to obtain far clearer insight into the role of the spleen in the transplantation immunity, two experiments were performed: one in which immune rats were irradiated with 500 R on the whole body excepting only the spleen, and the other in which only the spleen was exclusively irradiated with 500 R; and in both, the transplantation was made at 48 hours after the irradiation. In excluding the spleen, its site was determined by palpation in some, and by visual observation after exteriorizing it under anesthesia in others. Further, instead of exclusive irradiation of the spleen, splenectomy was performed in some. The results are shown in Table 3. It can be seen herein that in all the groups, the transplanted Yoshida sarcoma cells were perfectly rejected.

Table 3 PARTIAL X-IRRADIATION ON THE IMMUNIZED RATS III

IRRADIATED PART	EXTERIORIZING SPLEEN	NO. OF ANIMALS	NO. OF DEATH
WHOLE BODY EXCEPTING SPLEEN	WITH	6	0
	WITHOUT	2	0
SPLEEN ALONE	WITH	6	0
	WITHOUT	3	0
SPLENECTOMY INSTEAD OF IRRADIATION	———	3	0
NON-IRRADIATED CONTROL	WITH	3	0
	WITHOUT	6	0
UNTREATED CONTROL	WITH	6	6
	WITHOUT	6	6

Summary

Significant effect was not produced on the already acquired immunity whether the spleen of the immune rat was exclusively irradiated or splenectomy was performed. Likewise the immunity was not abolished when the spleen was excepted from the whole body irradiation. This means that although the spleen may play an important part as an organ of the antibody production system, neither its irradiation nor its excision produces any significant effect on the acquired immunity if the other part of the antibody production system is maintained intact.

II. Effect of irradiation on cellular antibody

Experiment 4.

The following experiments were performed to investigate transplantation immunity of the spleen and the lymphnode cells in immune rats:

Spleens and lymphnodes from immune rats were respectively smashed on double stainless net, and freed cells were suspended in Eagles medium solution. Each suspension was washed three times to remove fluid contained in the spleens or the lymphnodes. In preparing spleen cell suspension, heparin was added to prevent coagulation. To each cell suspension were added Yoshida sarcoma cells at the rate of 1 to 30 nucleated cells in the former. These original suspensions were diluted with the medium

solution to concentration of 10^7 Yoshida sarcoma cells per 1 ml, and after incubation at 36°C for 100 minutes, 0.1 ml was intraperitoneally injected to untreated rats.

As seen in Fig. 1, both the spleen and the lymphnode cells from immune rats inhibited proliferation of Yoshida sarcoma, whereas those from non-immunized healthy rats manifested scarcely any such inhibitory effect.

Summary

The presence of the anti-tumor action was demonstrated in the spleen and the lymphnode cells in immune rats. This will mean that cellular antibody is responsible for the anti-tumor immunity. At least above 90% of the lymphnode cells were small lymphocytes. There is consequently great possibility that the small lymphocytes may play an important role as the bearer of cellular antibody.

Experiment 5.

It was found by Exp. 4 that the spleen and the lymphnode cells in the immune rats had anti-tumor action. Now, investigation was made on effect of irradiation on lymphoid cells which had anti-tumor action. As lymphoid cells were used only lymphnode cells, which are considered not different essentially from spleen cells. These latter were not used since the spleen contains many other cells than lymphoid cells and further since heparin, which is known to have anticomplemental effect[10], has to be added in preparing spleen cells to prevent coagulation. We feared scatter in data owing to these mixtures.

Lymphnode cells from immune rats were placed in test tubes, and irradiated with 500R, and then the same procedure as in Exp. 4 was followed.

The results are represented in Fig. 2. The anti-tumor action was abolished in the irradiated lymphocytes.

Fig. 1 Anti-tumor action of immune lymphoid cells

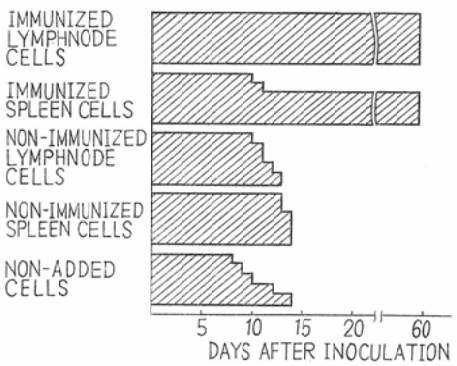
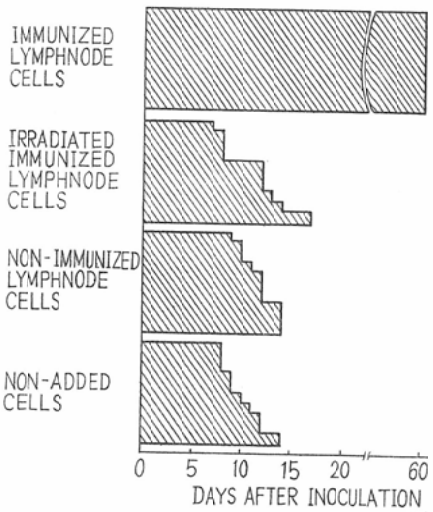


Fig. 2 Effect of X-irradiation on anti-tumor action of immune lymphoid cells



Summary

Lymphnode cells in immune rats lost the anti-tumor activity when irradiated with 500R, Considering the radiosensitivity, this is to be ascribed not to destruction of antibody molecule itself but to damage of cells.

III. Histological change in the spleen of immune rat by whole body irradiation

Experiment 6.

It is considered that production of antibody is performed almost entirely in the lymphoreticulocyte histological change of the spleen by irradiation, the major object will be attained in research of damage by irradiation of the antibody production system.

Immune rats, which received 500R whole body irradiation were sacrificed from time to time for histological examination during a period of 1 hour to 4 weeks after the irradiation. At 1 hour, destruction of lymphocytes was visible (Plate 1), and at 3 hours, the majority of them were either destroyed or flowed away, leaving scarcely any normal ones. The pyronin-positive large cells, which were extensively distributed in follicles and medullary cord before the irradiation, also became hardly visible, while strong pyroninpositive plasmocytes were seen around small blood vessels (Plate 2). At 6 hours, macrophages which had devoured a large amount of nuclear fragments were sporadically observed in follicles which had lost lymphocytes (Plate 3). Remmants of destroyed cells had almost been cleared away at 24 hours, and reticulocytes[11] as supporting tissues and sinal endothelium formed a net-like arrangement, with macrophages scattered here and there. The histological picture was thus monotonous (Plate 4). From this time on, weight of the spleen showed marked decrease, becoming on 2 days 1/3 as small as that before the irradiation. As for the histological picture in this period, reticulocytes were dominant (Plate 5). After 3 days some displayed a recuperative picture, but with considerable individual difference, advanced ones showing erythroblasts beneath the capsule and the pyronin-positive cells in the perivascular region (Plate 6). But it was 7 days and later that recuperative tendency of the follicle was visible. Some were not recovered perfectly even after 4 weeks. The spleen weight was at the lowest from 2 to 5 days after the irradiation, but tended to rise on about 7 days, approximately returning to the pre-irradiation level in 3 weeks.

Summary

Cells of the lympho-reticulocyte system, which are considered to produce antibody, were lost from the spleen within several hours after whole body irradiation with 500R. By contrast, cells of the plasmocyte system, especially mature plasmocytes, were relatively resistant to irradiation, and were found sporadically around the small blood vessel after irradiation.

It was about 2 days after irradiation that its damage is most intensively manifested by the spleen weight and histological picture. Later, the recuperative tendency became visible, but the lymphoreticulocyte system was restored latest, and some remained unrestored as late as 4 weeks.

Discussion

Rats which acquired resistance to Yoshida sarcoma lost it in 2 days when irradiated on the whole body with 500R (Report 1[9]).

In the present report it was disclosed that the anti-tumor resistance was abolished by irradiation of the lower half of the body containing the spleen. The resistance, however, remained undestroyed either when the spleen alone was irradiated, or when whole body irradiation was performed with protection of the spleen. Namely, the anti-tumor immunity could not be destroyed either without irradiating the spleen, or by irradiating the spleen alone. This means that the spleen is one of the most important organs of the antibody producing system, but not the only one, and that antibody may be produced without the spleen if the other part of the system, e.g. lymphnodes are preserved intact.

Plate 1 At 1 hour after irradiation, destruction of lymphocytes in follicles is visible. H. E. stain.

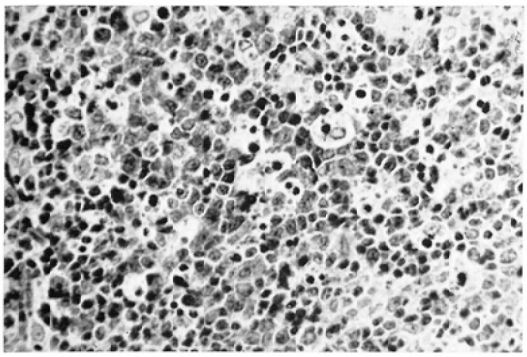


Plate 2 At 3 hours after irradiation, storong pyronin-positive plasmocytes are seen around small blood vessels. M.P. stain.

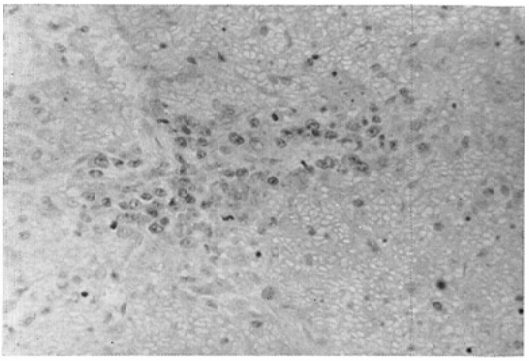


Plate 3 At 6 hours after irradiation, follicles have almost lost intact lymphocytes. H. E. stain.

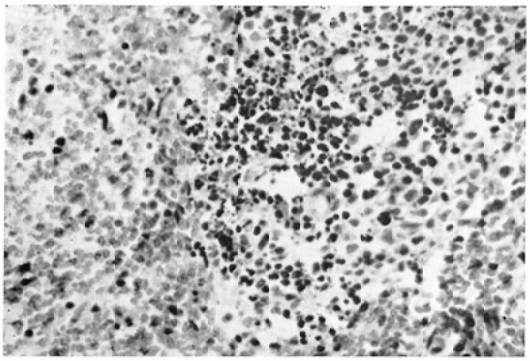


Plate 4 At 24 hours after irradiation, remnants of destroyed cells have been cleared away. H.E. stain.

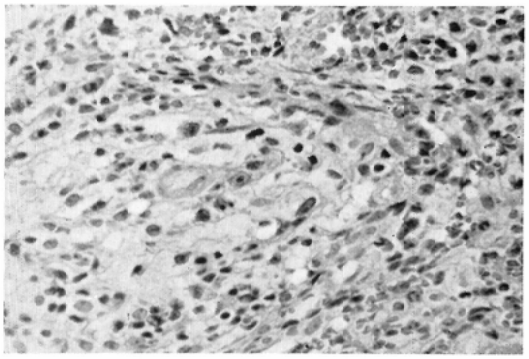


Plate 5 On 2 days, reticulocytes are dominant. H.E. stain.

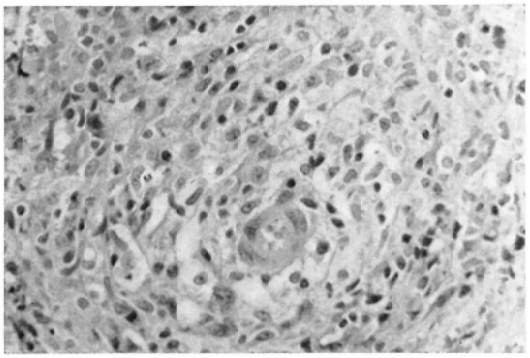
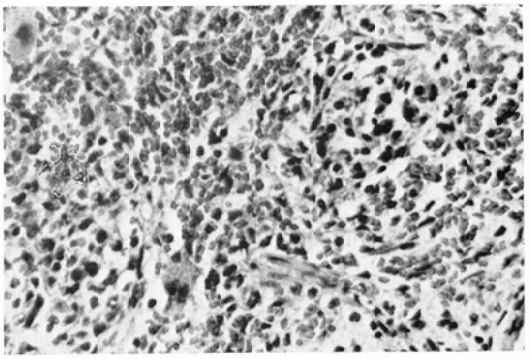


Plate 6 After 3 days, the pyronin-positive large cells are reappeared in the perivascular region and in the follicles. H.E. stain.



It is known that in transplantation immunity, cellular antibody, which is related with lymphatic system cells, is responsible[6)7)8)]. Concerning the immunity against the homotransplantation of Yoshida sarcoma, Itoh et al[12)] clarified that when there are disagreement of histocompatibility antigen, R-antigen, between the host animal and the tumor, humoral antibody is involved but that the principal part is played by cellular antibody related with the lymphatic system cells. Also the present experiment evidenced that the spleen cells as well as lymphnode cells in immune rats possessed the anti-tumor activity.

As for the mechanism of the abolishment by irradiation of the acquired anti-tumor activity, four possibilites can be considered: (1) Destruction of antibody molecules, (2) damage of lymphoid cells, (3) damage of the plasmocytes of lymphoid cells, and (4) damage of antigen or antigen information.

All know that a massive dose of radiation is necessary to elicit irreversible change in protein[13)]. There are also reports that no change can be produced in antibody molecules themselves by in vivo irradiation in sublethal dosis[14)15)]. It is therefore unconceivable that cellular antibody it self, much less serum antibody, should lose its activity on irradiation with 500R. The first possibility can thus be denied. The second possibility was supported by our experiment 5, in which the anti-tumor activity of lymphnode cells from immune rats was abolished by in vitro irradiation. Lymphnode cells are said to have higher radiosensitivity than any other cells of rats, the LD_{50} being 100R in vivo at 37°C. The lymphnode cell suspension used in the present experiments contained at least above 90% small lymphocytes, and 500R irradiation is assumed to have destroyed the majority of them. There is, however, question as to why the anti-tumor activity is abolished by destruction of lymphocytes in the test tube. By the fluorescent antibody method, Hanaoka et al[8)] elucidated that if the anti-tumor immunity is maintained by cell-bound antibody, it should remain in the test tube even after the destruction of the cell. This point will be explored further in our future studies.

Next we will consider damage to the blastocytes of lymphocytes, that is, pyronin-positive large sized cells[8)16)] in the spleen and lymph nodes. Histological examination of the spleen of rats irradiated on the whole body revealed that the majority of the pyronin-positive large sized cells were lost in several hours after irradiation, and tended to be restored on about 3 days. The perfect restoration, however, required more than 4 weeks. It is therefore doubtful whether the damage of the pyronin-positive large sized cells may have direct connection with the disappearance of the already existing antibody. But it is plausible that the damage of the cells may impede new production of antibody.

It was clarified in the first report that 500R whole body irradiation destroyed the transplantation immunity possessed by immune rats. But at the same time we reported that when Yoshida sarcoma cells were transplanted to these animals 9 days or more after the irradiation, some of them were cured despite transient proliferation of the tumor cells, whereas all the control rats died of the tumor when they received transplantation of Yoshida sarcoma cells without any previous treatment. It is therefore considered that although the anti-tumor activity in immune rats is lost by irradiation, it will intensively be acquired again on retransplantation of Yoshida sarcoma as an antigen. This suggests that the abolishment of the anti-tumor activity by irradiation may result not solely from damage of the antibody-producing cells but also from loss of antigen or antigen information. However, we can not advance our study further in this direction since it is obscure for the mcment what mechanism the antigen information has.

The above discussed can be summarized as follows:

Abolishment by irradiation of anti-tumor activity of immune rats can be attributed primarily to

damage of lymphocytes and their blastocytes and additionally to other still unknown factors. According to Everett et al, however, some of lymphocytes, irradiated in vivo, are enlarged, showing DNA synthesis or nuclear division. If so, there may be change of lymphocyte-lymphoblast, and subsequently it is not necessary to consider damage of lymphocytes separately from that of their blastocytes (lymphogonia).

There is concensus of opinion in that the antibody production system consists of plasmocyte and lymphocyte systems. Mitsuhashi et al[18)19)] consider that in experimental typhus, intraperitoneal mononuclear cells also produce antibody. Comparison of radiosensitivity between these three systems revealed that it was high in all the cells of lymphoreticulocyte system and also in the blastocytes of plasmocyte system, but was low in mature plasmocytes and intraperitoneal mononuclear phagocytes[20)]. Therefore the fact that the anti-tumor activity of immune rats was abolished by 500R irradiation may indicate that the lympho-reticulocyte system plays the principal role in the transplantation immunity.

Conclusion

In order to elucidate the mechanism by which resistance to Yoshida sarcoma of immune rats is abolished by irradiation, radiosensitivities of different kinds of cell which are considered to produce antibody were examined.

1. When immune rats were irradiated on the lower half of the body containing the spleen, the anti-tumor activity was lost, but when the whole body irradiation was made with the spleen protected, the resistance remained. Exclusive irradiation of the spleen, however, did not produce loss of the resistance.

2. It is the lympho-reticulocyte system which plays the principal role in the transplantation immunity. The majority of them were damaged by 500R irradiation, and it took several weeks for them to be restored.

3. The spleen and the lymphnode cells were considered to have intimate relation with cellular antibody, since when these cells were damaged, the anti-tumor activity was also abolished concurrently.

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