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(Citation)
The Kobe journal of the medical sciences,10(4):265-276

(Issue Date)
1964

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100489186

Kobe J. Med. Sci.
10, 265-276 , Dec. 1964.

IMMUNE RESPONSE OF EHRLICH ASCITES TUMOR CELLS IRRADIATED *IN VITRO*

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H. Green, B. Goldberg and their associates,[1)-6)] and K. A. C. Ellem[7)] have demonstrated by electron microscopic and biochemical studies that the primary action of antibody and complement on animal cells is the production of a permeability defect in the cell membrane leading to a form of colloid osmotic lysis. In addition, it was pointed out by us[8)] that antibodies to the cell components penetrate into the interior of cells across the membrane, following the formation of functional holes in the membrane, which will be reported elsewhere.

While, there have been appeared some reports that irradiation also inflict serious damage on the cell membrane.[9)-12)] P. Alexander even stated that they had to reject the view, basing on the radiochemical studies, that damage to DNA protein constituted the primary lesion at least for the process leading to cell death. Instead of that, he postulated that the alteration of the permeability of the cell membrane had to be taken into consideration as the primary site of radiation damage.[12)]

Then, it would seem reasonable to suppose that the irradiated mammalian cell would show different response to antibody from that of the non-irradiated; the difference would afford a promising clue for elucidating the mechanism of radiation injury on mammalian cells. It would also supply a interesting problem on the radiation therapy of malignant tumors from the practical point of view.

The present work deals with the immune response of irradiated Ehrlich ascites tumor cells exposed to anti-EAT gamma globulin *in vitro*.

MATERIALS AND METHODS

Tumor Cells: Ehrlich ascites tumor (EAT) cells were harvested from the peritoneal cavity of adult mice inoculated 3 days previously with 0.1 ml of tumor bearing ascites fluid. After separation of cells from the ascites fluid by centrifugation, the removed cells were suspended in 10.0 ml of Eagle's balanced salt solution (BSS) at PH 7.4,[13)] containing 1 mg of glucose per ml. After recentrifugation, the supernatant fluid was decanted and cells were resuspended in 3.0 ml of BSS to yield a concentration of approximately 2 X 10^7

Received for publication Dec. 14, 1964
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cells per ml, each 1.5 ml aliquot of this suspension was transfered to a small test tube Cell counts were performed with a hemocytometer.

Antibody and Complement: Washed whole tumor cells from 0.5 ml of ascites fluid were injected subcutaneously into rabbit once a week for at least 4 weeks with equal volume of complete Freund adjuvant. Ten days after the last injection, the rabbits were bled, the sera were separated and stored at —20°C after proving formation of the anti-EAT cells antibody by gel diffusion technique,[14] on which usually three distinct precipitation lines were confirmed against anti-EAT sera and no line against serum taken before immunization. Rabbit gamma globulin was prepared from the serum of the immunized animals according to the salting out method, described by Humphrey and McFarlane,[15] using sodium sulfate. As the source of the complement, lyophylyzed guinea pig sera commercially available (Toshiba Serum Lab.) were used in a 1 : 10 dilution of the original serum. Inactivated complement was prepared by heating the serum at 56°C for 30 minutes.

Labeling of Gamma Globulin with ^{131}I: Gamma globulin was labeled with ^{131}I according to the method of Wissler *et al.*[16] Uncoupled iodine and isoluble protein were removed by passing the protein solution through the column of Amberlite IR-4B (OH-form) followed by 10 ml of distilled water.[17] The eluate which contained labeled gamma globulin was dialyzed against a bulk of saline buffered at PH 7.4 overnight. Specific activity of ^{131}I per mg protein varied to some extent from specimen to specimen.

Fractionation of Cell Components: The EAT cells homogenized by Potter-Elvehjem type of homogenizer were fractionated by differential centrifugation with Spinco L ultracentrifuge according to the method of Hogeboom and Schneider.[18]

Radio-iodine and Counting Procedure: ^{131}I was supplied from Japan RI Society as carrier-free ^{131}I. The specific activity of specimens were counted routinely for 5 minutes with a well-type scintillation counter (Shimazu Co. Ltd.), and the average counts were expressed as counts per minute (cpm).

^{131}I-Serum Albumin: Commercially available human ^{131}I-serum albumin (RISA, Abbot Lab.) was used throughout. The original solution containing 10 mg albumin per ml and 1 mc per ml was diluted 10 times with distilled water. It was supplemented with 30 micro gram per ml of streptomycin and 40 micro grams per ml of penicillin and stored at 0°C.[19]

FINDINGS

The viability of cells were examined by staining them with crystal violet or nigrosin[20] [21] at various time intervals during incubation. Furthermore, a number of samples were fixed with buffered osmium tetroxide for electron microscopic examination. In order to find an adequate does of ^{60}Co gamma rays to be used, effects of irradiation alone on the viability of the cells were examined with various doses such as 50, 100, 300, and 500 r. The result is shown in Fig. 1. Percent survivals after 90 minutes incuba-

tion were 90% at 100 r and 84% at 500 r. respectively. Per cent survival were calculated according to the method of Aoyama.[22] Since dead cells would indicate altered permeability, corrections for these death rates were made hereafter in all the experiments through the present work.

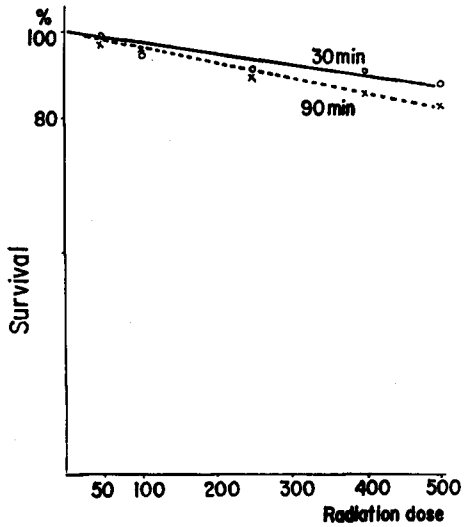

Fig. 1.
Survival curves after irradiation. Per cent survival 30 and 90 minutes after several irradiations with serial does of gamma-rays.

I. Effect of irradiation on the permeability of immune gamma globulin

This experiment was performed with the purpose of finding the membrane defect which might be produced after irradiation. The experimental system is summarrized in Table 1. Washed cell suspensions were irradiated with 500 r of ^{60}Co gamma rays.

Table I

I^{131} labeled anti whole cell gamma globulin (3.0 mg/ml)	1.5 ml
Washed cell suspension (2×10^4 cells/ml)	1.5 ml
Eeagle's balanced salt solution	2.5 ml

Table 1. Washed cell suspension was irradiated with 500 r of ^{60}Co gamma-rays before incubation. Incubation was carried out at 37°C for 90 minutes with continual agitating. Non-irradiated cell snspension was treated in the same way, which served as control. Instead of ^{131}I-labeled gamma globulin, normal rabbit serum gamma globulin, RISA were used.

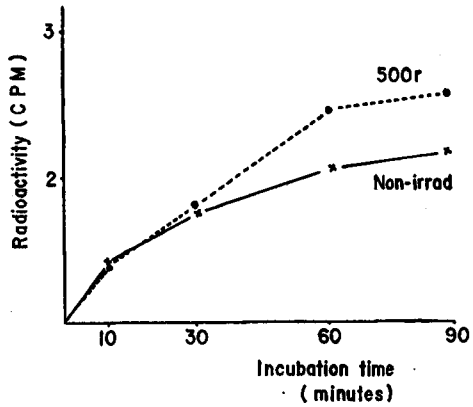

Fig. 2. Take up of labeled immune gamma globulin. The radiactivity of whole cell pellet. Influence of irradiation become demonstrable after 30 minutes' incubation. The radioactivity in the ordinate is expressed as the ratio of CPM at the gievn time to CPM at the 0 time.

Immediately after irradiation, they were added with ^{131}I labeled anti-whole cell or anti-mitochondrial gamma globulin and BSS, and incubated for 90 minutes at 37°C under incessant agitating. Non-irradiated cell suspensions were treated in the same way, which served as control. At appropriate time intervals, such as 0, 10, 30, 60, and 90 minutes, an aliquot of 1.0 ml was withdrawn from the incubation mixture, immediately chilled in the ice-bath diluting with two volumes of cold BSS, centrifuged for 5 minutes at 4°C and and 3000 rpm, and the washing was repeated twice with 2.0 ml of cold BSS. Then, the radioactivity remaining in the pellet was counted as mentioned previously.

The result is illustrated in Fig. 2. In the ordinate of the figure, the radioactivity of whole cell pellet is expressed as the ratio of cpm at the given time to cpm at the 0 time, that is, as the ratio of increase in radio-activity calculated as an average of at least five experiments. Influence of irradiation became demonstrable after 30 minutes incluba-tion and the irradiation doses had not so much influence on the rate of increase in radio-activity.

When the normal rabbit serum gamma globulin was used instead of anti-EAT gamma globulin, the effect of irradiation has not been demonstrated, as seen in Fig. 3. But, when RISA was used, the take up of radioactivity by whole cell pellet was again clearly exhibited. From these data, it may be deduced that the irradiated cells takes up more anti-whole cell EAT immune gamma globulin than the non-irradiated and this effect of irradiation on the take up of macromolecules is exhibited only against immune gamma globulin or RISA, but not against normal gamma globulin, the reason of which will be discussed later.

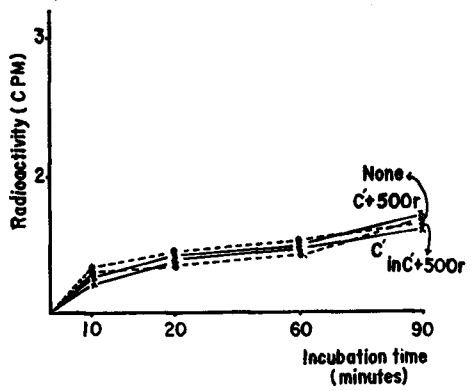

Fig. 3. Take up of labeled normal gamma globulin. The radioactivity in whole cell pellet of ^{131}I-labeled normal rab-bit serum gamma globulin. The effect of irradiation was not demonstrated and not changed in the presence or absence of complement.

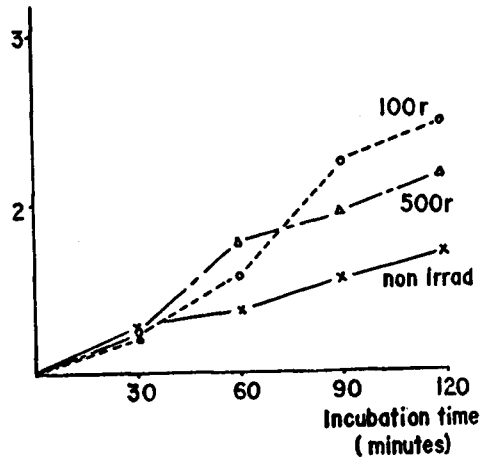

Fig. 4. Take up of labeled human serum albumin. The uptake of albumin was clearly exhibited by irraditation.

Radioactivity remaining in the pellet after three washings, however, consists of those fractions such as adsorbed on the cell membrane by unspecific physico-chemical

forces, combined with the cell membrane by immune reaction and penetrated into the interior of cells across the membrane. So, the radioactivity in these fractions must be analysed to confirm the modificatory action of radiation on permeability of the cells. In order to carry out the analysis, the pellet treated by ^{131}I labeled anti-EAT immune globulin was homogenized immediately after counting and centrifuged differentially by ultracentrifuge. Fractions named nuclear, membraneous, mitochondrial. microsomal and sap, were counted

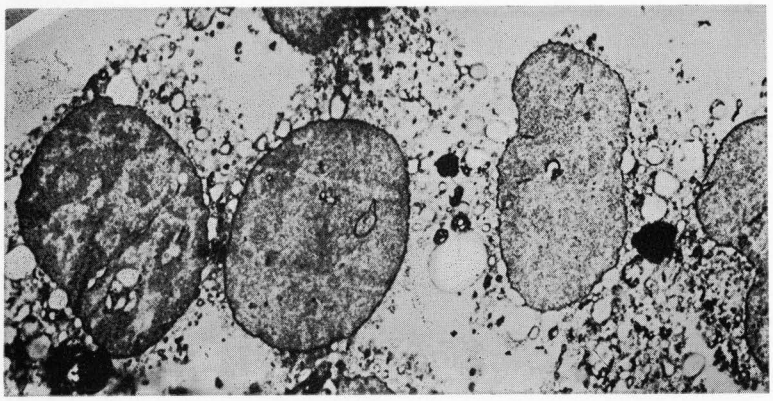

Fig. 5. Isolated nuclear fraction.
The nuclear fraction isolated by this procedure is contaminated with residual intact cells, or other cell components.

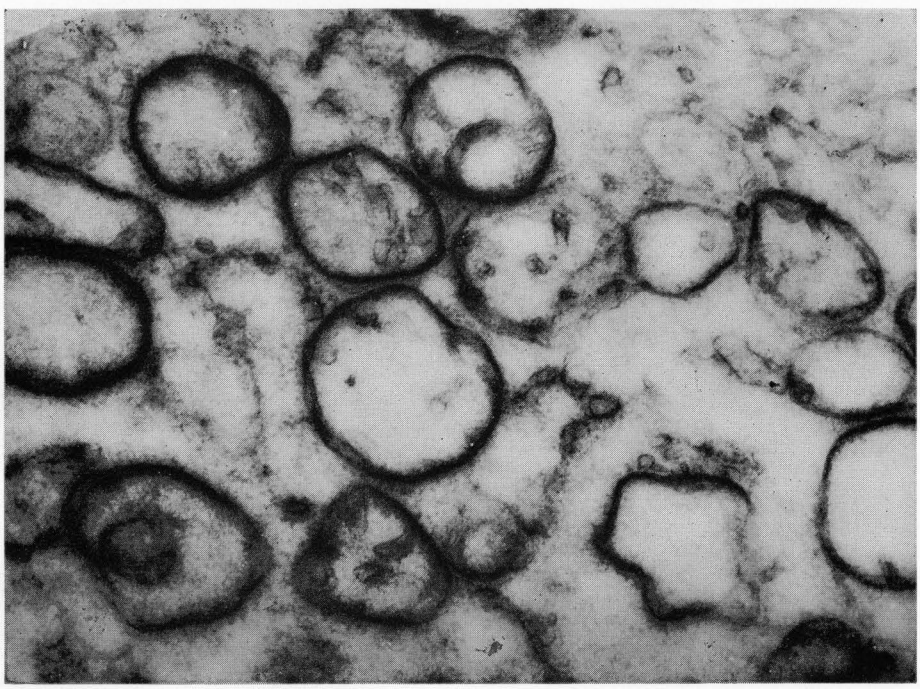

Fig. 6. Isolated mitochondria.
The mitochondrial fraction is reasonably pure on the basis of cytological criteria.

on their radioactivity with a well-type scintillation probe. Slight contamination was found inevitably of each other fraction under the electron microscopic pictures, which were illustrated in Fig. 5, and 6.

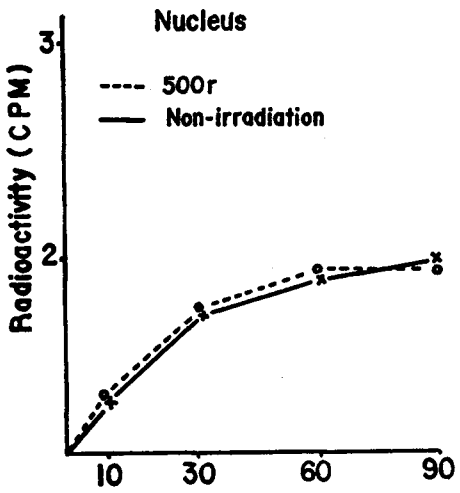

Fig. 7. Take up of labeled immune gamma globulin.
The radioactivity of nuclear fraction. No particular difference from control was seen.

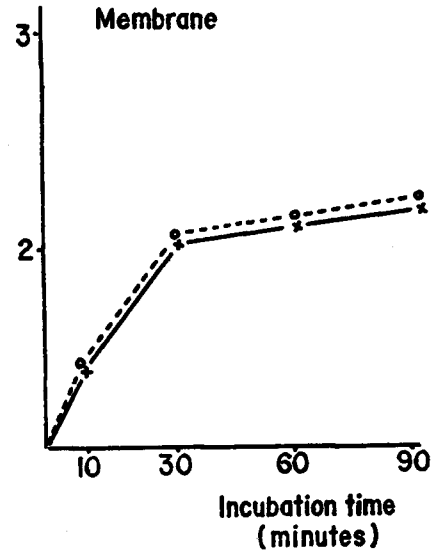

Fig. 8. Take up of labeled immune gamma globulin.
Nembranous fraction. No particular difference from control was seen.

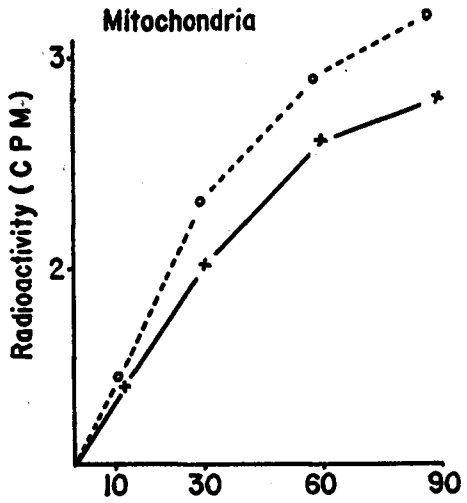

Fig. 9. Take up of labeled immune gamma globulin.
Mitochondrial fraction. The significant difference of radioactivity between the irradiated and the non-irradiated was noticed.

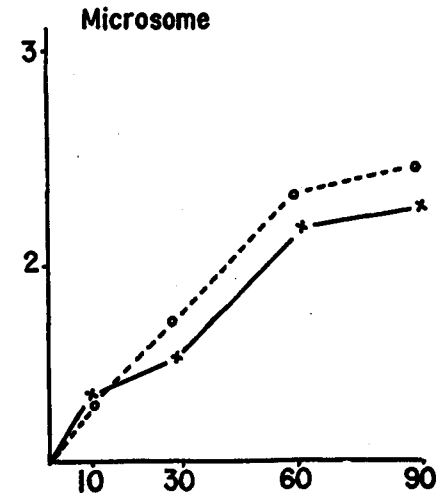

Fig. 10. Take up of labeled immune gamma globulin.
Microsomal fraction. The significant difference of radioactivity between the irradiated and the non-irradiated was noticed.

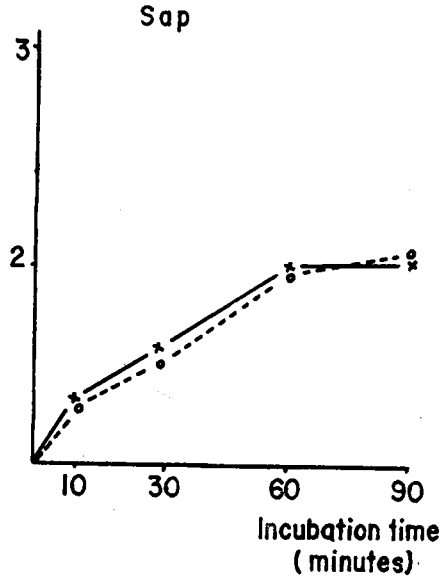

Fig. 11. Take up of labeled immune gamma globulin.
Cell sap. No particular difference from control was seen.

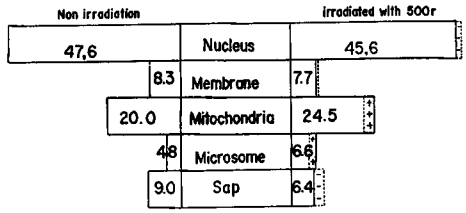

Fig. 12. Per cent radioactivity of fraction.
Per cent radioactivity of each fraction to that of whole cell pellet irradiated with 500 r (incubation time; 60 minutes). In irradiated group, the radioactivity of nucleus, membrane and sap is decreased and that of mitochondria and microsome is increased.

Among the five fractions differentiated, mitochondrial and microsomal fractions showed the significant difference of radioactivity between the irradiated and non-irradiated. Other three fractions were almost eaqual in increasing radioactivity. (Fig. 7, 8, 9, 10, 11, 12)

Because of the contamination of fractions in each other, seen in electron micrographic pictures, quantitative expression can not be so highly relied upon, but it might be semiquantitatively stated that the immune gamma globulin could penetrated into the interior of cells more in the irradiated than in the non-irradiated cells.

Since the mitochondrial fraction demonstrated the significant difference of radioactivity between irradiated and non-irradiated cells with anti-whole cell antibody, anti-mitochondrial immune gamma globulin was tested in the same way, but no difference in penetration was observed between the two. It may be considered that the antibody to the surface membrance of cells is responsible for the higher penetration of macromolecules by irradiation.

II. Radiation-induced permeability changes of anti-whole cell immune gamma globulin in the presence or absence of complement.

In order to examine the permeability changes by irradiation in the presence or absence of complement, experiments of two systems were carried out, which were summarized in Table 2.

Table 2

System I (Incubation for 90 minutes)	Curve R_1	Curve R_2
Washed cell suspension irradiated with 500 r (2×10^4 cells per ml)	1.5 ml	1.5 ml
Complement (7.5 dilution)	2.0 ml (active)	2.0 ml (inactive)
^{131}I labeled immune gamma globulin (3.0 mg per ml)	1.5 ml	1.5 ml

System II (Incubation for 90 minutes)		Curve R_3	Curve R_4
Washed cell suspension (2×10^4 cells per ml)	Irradiated with 500 r	1.5 ml	1.5 ml
Complement (7.5 dilution)		2.0 ml (active)	2.0 ml (inactive)
^{131}I labeled immune gamma globulin		1.5 ml	1.5 ml

*Non-irradiated systems serve as control in all the experiments (Curve R_5, R_6).

That is, in System I, only the washed cell suspension has been irradiated, while, in System II the washed cell suspension and complement, active or inactive, have been irradiated simultaneously, and the non-irradiated system serves as control.

The rseults are illustrated in Fig 13 and 14, and summarized in Fig 15, in which per cent radioactivities of whole cells at 60 minutes of incubation in various experimental conditions are indicated, taking the values of Curve R_5 (non-irradiated cells+Ab+active C') as 100%.

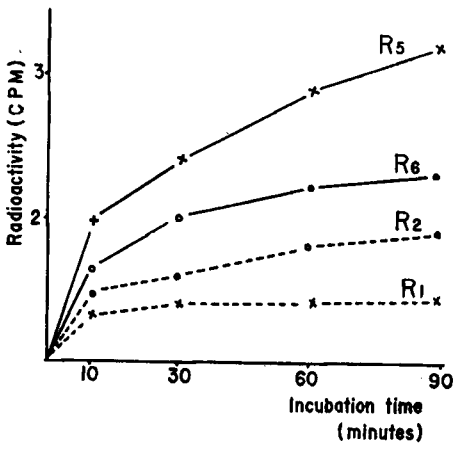

Fig. 13. Take up of labeled immune gamma globulin.
The result of System 1. Solid line shows the radioactivity of non-irradiated cell pellet and dotted line shows that of irradiated cells (Complement non-irradiated).

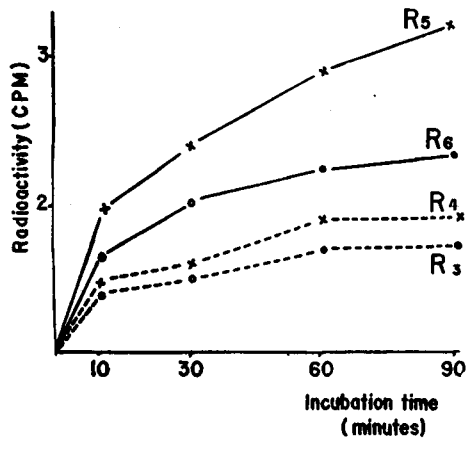

Fig. 14 Take up of labeled immune gamma globulin.
The result of System 2. Solid line shows the radioactivity of non-irradiated cell pellet and dotted line shows that of irradiated cells (Complement also irradiated together).

From these figures and the previous results, it may be concluded that: (1) when non-irradiated cells were used, the permeability of cells is enhanced by antigen-antibody reaction in presence of active complement, but not in presence of inactivated complement (Compare Fig 2 with Fig 4), (2) while, with irradiated cells, permeability rather decreased by antigen-antibody reaction in presence of active or inactive complement (compare R_5 and R_6 with R_1, R_2, R_3 and R_4), (3) active complement is supressed by irradiation (inactivation by irradiation), while heat-inactivated complement is not affected by irradiation (compare R_2 with R_4 and R_1 with R_3).

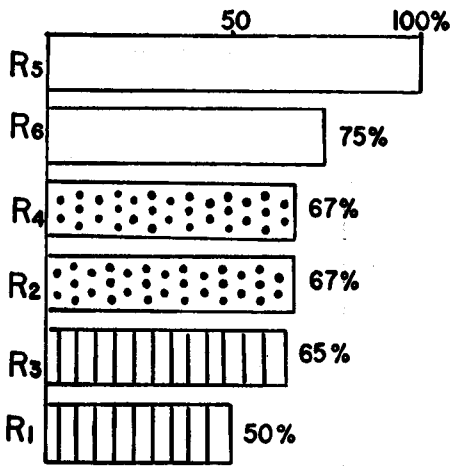

Fig. 15. Per cent radioactivities of whole cells at 60 minutes of incubation in various experimental conditions are indicated, taking the values of curve R as 100%.

DISCUSSION

It is assumed by some investigators that primary site of irradiation damage might be the cell membrane. N. Doemin and V. Blokhina[23] demonstrated the biochemical damages of lipids in cell membrane after irradiation, and P. Alexander[12] reported that anucleated half of *Amoeba proteus* also show the typical radiation lesion after irradiation. If this were the case, it would be reasonable to assume that permeability of cell membrane would also be altered by irradiation.

In the first part of the report, it was clearly demonstrated that the membrane permeability of cells have been changed by irradiation and more immune gamma globulin penetrated into the interior of the cell across the membrane. In order to cause higher passage of macromolecules, antimembrane immune globulin is necessary, because with anti-mitochondrial globulin no difference was demonstrated between the irradiated and non-irradiated cells. Therefore, irradiation would have caused the biochemical or functional changes in the membrane, by which it would become vulnerable to anti-membrane immune gamma globulin.

As for the difference of taking up anti-whole cell immune gamma globulin among cell components, it is easily explained by the difference of antigenicity among them.[24] In fact, E. Horn[25] reported poor antigenicity of nucleus. The anti-whole cell immune gamma globulin which was used in the present work demonstrated three clear lines by Ouchterlony gell diffusion technique against homogenized whole cell suspension, one against cell membrane, against mitochondrial fraction and the rest probably against microsome or endoplasmic reticulum, but it does not necessary mean the tumor specificity, to

which caution must be taken.

Secondly, it is morphologically or biochemically demonstrated by many investigators [1)-6) 26)] that immune reaction can cause a membrane defect in the presence of active complement, and give rise to a functional hole in the cell membrane, through which many small ions and nucleotides escape. Green *et al* named the phenomenon immunocytolysis. von Ellem[7)] also reported the vulnerability of cell membrane by antigen-antihody reaction in pressence of complement. This immunocytolysis is also confirmed by us and a part of immune gamma globulin enters the cells across the membrane which will be reported elsewhere. So, I have assumed that irradiated cell would be more vulnerable to immune cytolysis. According to the second part of this work, this is not the case. Complement worked on the opposite direction, that is, generally, immune lysis is accelerated by complement, or does not occur without it. While with irradiated cells, on the contrary, complement protect the cells from immune cytolysis and active complement is stronger in the effect than the inactivated complement. Furthermore, it was demonstrated that complement can be inactivated by irradiation. This might be due to that functional holes in membranes caused by irradiation had been protected by complement molecule, or that irradiation had removed certain component in membrane, which would be acted on by esterase-like activity of complement.[27)] The precise mechanism remains to be studied further.

Thirdly, it should be taken into consideration what a sort of permeablility is dealt with at the present work. As the mechanism of penetration of substances across the membrane, four cases are generally known: simple diffusion, active transport, pinocytosis and phagocytosis.[28) 29)] Since the labeled gamma globulin was used in our experiment, the mechanism of permeability would be reasonable to assume to be pinocytosis. According to our preliminary experiment, it is considered that phagocytosis is supressed by irradiation; and other small isns are indefinite according to the kinds of ions.[30)] Therefore, when permeability of a substance is discussed, the size or nature of the substance must be taken into consideration,[31)] and in our case, large size of immune gamma globulin, about 160,000 of molecular weight, have been the object of discussion.

It would be beyond our expectation that this work would ccntribute to the fundamentals of radiation therapy of malignat tumors.

SUMMARY

(1) Permeability of cell membrane to large molecules was increased by irradiation.
(2) Immune cytolysis was protected by irradiation.
(3) Complement coul be inactivated by irradiation.
(4) The mechanisms was discussed.

AKNOWLEDGEMENT

The author is grateful to Prof. K. Narabayashi, Department of Radiology and Prof. C. Endoh, Director of Growth Research Institute. The author expresses his sincere thanks to Associate Prof. T. Kondoh, Prof. I. Suda, Prof. S. Hotta, and Associate Prof. S. Kimura, Growth Research Institute, for their guidance, incessant encouragement, discussion and criticism during the present work, and also to Dr. T. Aoyama, and Dr. Y. Fujiwara, Growth Research Institute, for preparation of materials for this investigation. Aknowledgement is also due to Mr. Y. Kawamoto and Mr. M. Yoshino, Growth Research Institue, for their technical assistance.

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