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美洲商陆丝裂原(PWM)和脂多糖(LPS)激活的淋巴细胞的相互关系及其辐射敏感性

THE RELATIONSHIP BETWEEN LYMPHOCYTES ACTIVATED BY POKEWEED MITOGEN AND BY LIPOPOLYSACCHARIDES AND THEIR RADIOSENSITIVITY

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摘　要

人血体外培养,分别用美洲商陆丝裂原和脂多糖激活,用放射性标记化合物掺入法测定两种细胞的相互关系。实验显示了PWM激活的淋巴细胞具有促进LPS对B淋巴细胞的激活效应。当PWM激活的淋巴细胞受10 Gy ^{60}Co γ射线照射后,这种促进作用明显减小。当PWM和LPS激活的淋巴细胞共同培养时,如果其中一种事先受10 Gy ^{60}Co γ射线照射,其^{3}H-TdR掺入即显著降低,反映协同功能消失,尤其是PWM激活的淋巴细胞受照射影响更严重。鼻咽癌病人受^{60}Co γ射线治疗后,LPS激活的淋巴细胞掺入接近正常水平,而PWM激活的淋巴细胞掺入则明显降低,PWM激活的淋巴细胞对LPS激活的淋巴细胞的刺激效应亦明显减小。本文提示PWM激活的淋巴细胞具有T辅助细胞的功能,它比LPS激活的淋巴细胞辐射敏感性高。

THE RELATIONSHIP BETWEEN LYMPHOCYTES ACTIVATED BY POKEWEED MITOGEN AND BY LIPOPOLYSACCHARIDES AND THEIR RADIOSENSITIVITY

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ABSTRACT

Human whole blood was incubated *in vitro*. Lymphocytes were activated by pokeweed mitogen (PWM) and by Lipopolysaccharides (LPS). The relationship between the two kinds of lymphocytes was investigated using radioactive compound incorporation. The study showed that PWM-activated lymphocytes were able to promote the stimulating effect of LPS on B lymphocytes. The stimulating effect of PWM-activated lymphocytes was obviously decreased after they were irradiated with 10 Gy gamma rays. When PWM-activated lymphocytes and LPS-activated lymphocytes were incubated together after one of the cell populations had been exposed 10 Gy ^{60}Co gamma rays, the incorporation of [^{3}H] TdR was much decreased and the synergistic function disappeared, especially when the PWM-activated lymphocytes were irradiated. In cells from patients treated with ^{60}Co gamma rays for carcinoma of nasopharynx, the incorporation in LPS-activated lymphocytes approached normal levels while that in PWM-activated lymphocytes was reduced significantly and the stimulating effect of PWM-activated lymphocytes on LPS-activated lymphocytes was also markedly reduced. These demonstrate that PWM-activated lymphocytes have a similar function to T-helper cells and seem to be more radiosensitive than LPS-activated lymphocytes.

INTRODUCTION

Lymphocytes are important part of the immune system of an organism. There is not a single population but a number of subpopulations of lymphocytes which vary in their sensitivity to radiation with some more radiosensitive than others[1~15]. However, the secondary effects of irradiation of one subpopulation of lymphocytes on other subpopulations are not known. Further more, it is also not known if the relationship between two subpopulations is altered when both are irradiated, or if there is a differential effect between the two subpopulations. Studies of these aspects are rare. After radiation therapy, it is important to study the changes in the function of every subpopulation of lymphocytes and the relationship between them.

Pokeweed mitogen (PWM) has been shown to activate T helper cells, causing B cells not treated with PWM to secrete immunoglobulin[16,17]. The polyclonal immunoglobulin in B cells induced by PWM has been found to be highly reproducible, and the response is dependent on T cells[18,19]. The activation of OKT_4 cells by PWM has also been demonstrated[20]. Pokeweed mitogen has been shown to induce T helper cells in co-culture and B cells in single culture[21].

Lipopolysaccharides (LPS) were originally thought to be specific stimulants for B cells in the mouse[4,22]. However, several investigators failed to find stimulation of human peripheral blood lymphocytes (PBL) by LPS[14,23~26], although Miller et al. have reported the successful stimulation of human PBL by LPS[27]. In our experiments, under optimal conditions of incubation, we have been able to stimulate human PBL with LPS and to study the function of the human PBL T helper cell-B cell system and its response to irradiation. In this paper, the effect of ^{60}Co irradiation on human PBL T helper cell-B cell system activated by PWM and LPS and on this system of cancer patients after radiation therapy *in vivo* are reported.

Lymphocyte subpopulations are heterogeneous in that they contain both radiosensitive and radioresistant fractions.

In vitro studies it was demonstrated that human T suppressor cells were radiosensitive compared to T helper cells[3,5,14,29]. In one study, PHA-activated OKT_4 cells were irradiated with 30 Gy, and a marked increase in the response of Ig-secreting cells was seen[13]. In present study we have used 40 Gy gamma rays to irradiate PWM-activated

lymphocytes to determine whether the helper activity can be totally suppressed by gamma rays. We have also used 10 Gy gamma rays to irradiate PWM-activated or LPS-activated lymphocytes to determine if there is a change in function in single culture or co-culture.

1 MATERIALS AND METHODS

1.1 Human PBL

Blood was obtained from healthy volunteers by venipuncture and was heparinized. In patients with nasopharyngeal cancer the venous blood was obtained after the last radiation treatment.

1.2 Cell Culture and Mitogenic Stimulation

Heparinized whole blood was cultured in three different media :

PWM culture: Heparinized human whole blood (0.35 mL) was incubated at 37℃ in 3 mL Eagle's medium with 5 μg/mL PWM (Sigma Co.) and 10% AB pool serum. After 72 h, mononuclear cells from whole peripheral blood were isolated by centrifugation in Ficoll-Hypaque medium (Shanghai Second Chemical Factory), then were washed with saline and culture medium without the mitogen. These cells were identified as PWM cells.

Culture without mitogen: Heparinized human whole boold (0.35 mL) was incubated at 37℃ in 3mL culture medium with AB serum as described above except without addition of mitogen. After 72 h, mononuclear cells were separated and washed as described above. These cells were identified as control cells.

LPS culture: Human heparinized blood was separated and washed as described above and incubated in 2 mL Eagle's culture medium (2 mL) containing 20 μg/mL LPS (Difco Co.). A certain amount of mononuclear cells was added and incubated at 37℃ for 72 h. These cells were identified as LPS cells.

1.3 Irradiation

(a) Some cultures of PWM cells were exposed to 40 Gy ^{60}Co gamma rays (dose rate 4 Gy/min, distance 66 cm).

(b) Some cultures of PWM cells and LPS cells were exposed to 10 Gy ^{60}Co gamma rays.

(c) Six patients with nasopharyngeal cancer were treated with ^{60}Co gamma rays. The total doses of radiation for them were 93, 150, 154, 157, 159, and 164 Gy respectively.

1.4 Co-culture

(a) PWM cells and control cells were added to the LPS culture medium containing LPS cells for secondary culture as follows:

LPS cells+control cells;

LPS cells+PWM cells;

LPS cells+PWM cells irradiated with 40 Gy.

The following cells were cultured in LPS medium alone as controls:

LPS cells cultured continuously in LPS medium;

PWM cells;

PWM cells irradiated with 40 Gy;

Control cells.

(b) PWM cells, LPS cells, PWM cells irradiated with 10 Gy and LPS cells irradiated with 10 Gy were singly cultured or co- cultured in all appropriate combinations.

(c) PWM cells and LPS cells from patients and from normal individuals were incubated in all possible combinations.

In all experiments the number of LPS cells, PWM cells, irradiated PWM cells, irradiated LPS cells and control cells per bottle was $5\times10^5\sim10^6$, but the number of each kind of cells per bottle from the same individual was the same. These cells were incubated at 37℃ in 2 mL Eagle's medium with 10% AB pool serum.

1.5 [^{3}H]TdR and [^{14}C]UR Incorporation Assays

[^{3}H]Thymidine (SP act 70.3×10^{10} Bq/m mol, 3.7×10^4 Bq/mL of culture) and [^{14}C]UR (SP act 203.5×10^7 Bq/m mol, 0.555×10^4 Bq/mL of culture) were added to label DNA and RNA, respectively, after cultures had been incubated for 48 h. After a labeling period of 24 h, the mononuclear cells were collected on glass fiber discs, washed with distilled water and saline, fixed with 5% trichloride acetic acid and finally bleached with anhydrous ethanol. The disc was dried for 30 min at 60℃ and was put into 5 mL scintillation fluid containing 0.4% PPO and 0.04% POPOP. The amount of radioactivity of the incorporated [^{3}H]TdR and [^{14}C]UR was measured by liquid scintillation counting and expressed in counts per minute (cpm). The number of cells in different bottles varied and the results of the average incorporation of [^{3}H] TdR and [^{14}C]UR in cells of 12 duplicate cultures were normalized to cpm/5×10^5 cells per culture. A duplicate culture was set up and the average result was calculated. Student's test was used for statistical

analysis.

To avoid damage to lymphocytes by the separation and washing, whole blood was incubated in a PWM culture medium. Then separation was done after the first stimulation of the secondary culture. The LPS cells were cultured as separated lymphocytes so further separation was not needed. The incorporation of [^{3}H]TdR in separated lymphocytes was about 90% of that in whole blood lymphocytes when they were incubated in the same mitogen and under the same conditions.

Incubation time was set to allow the maximum effect of the mitogen. This was indicated by the time required to obtain maximum incorporation of [^{3}H]TdR.

The concentrations of PWM and LPS we used have been used by other investigators [16,23]. When we used PWM at a concentration of 5 μg/mL in the medium, the mean value of incorporation of [^{3}H]TdR in 5×10^5 lymphocytes reached 8994 cpm--a suitable level of counts for comparison with those obtained in other experimental groups. Ling used a concentration of 10 μg/mL LPS [21]. However, in our experiments, 20 μg/mL LPS was more effective than 10 μg/mL and 30 μg/mL, so these concentrations were selected in the present study.

2 RESULTS

2.1 The Change in the Function of PWM-Activated Lymphocytes after Exposure to Gamma Rays *In Vitro*

Table 1 The Effect of PWM-Activated Cells on Incorporation [^{3}H]TdR and [^{14}C]UR into LPS-Activated Cells (cpm/5×10^5 cells) (mean ±SE)

Group	[^{3}H]TdR	Percentage of control	[^{14}C]UR	Percentage of control
LPS cells+control cells	2219±306	100.0	1346±142	100.0
LPS cells+PWM cells	19363±1989***	872.6	6032±990***	448.1
LPS cells+PWM cells after exposure	3210±202	144.7	1391±89	103.4
LPS cells	1227±152		524±51	
PWM cells	8994±1571		2053±400	
Control cells	657±77		466±105	
PWM cells after exposure	2641±372		805±150	

$n=12$

*** $P<0.001$ vs. LPS cells+control cells.

The effects of cells activated by PWM before and after irradiation on transformation of human blood lymphocytes stimulated by LPS were determined using an isotope incorporation method. The results were expressed in cpm incorporated in 5×10^5 cells per culture. The average values of 12 tests are shown in Table 1.

The standard used for evaluating the effects of interactions of cells was the incorporation of [^{3}H]TdR and [^{14}C]UR in LPS and control cells expressed as cpm, which was considered as 100%. In comparison, the incorporation levels of DNA and RNA in LPS cells+PWM cells were 873% and 448% respectively. In contrast, the incorporation in LPS cells+PWM cells after exposure to radiation were 145% and 103%, respectively. The stimulating effect of PWM-activated cells was decreased after exposure to 40 Gy gamma rays by about a factor of 6 and 4, respectively.

When PWM-activated cells and LPS-activated cells were incubated together, the incorporation of [^{3}H]TdR and [^{14}C]UR was higher than the total incorporation when the two kinds of cells were incubated separately. This demonstrated the synergistic effect of the two kinds of cells. When LPS cells and control cells were incubated together, the incorporation of [^{3}H]TdR and [^{14}C]UR was the same as the total incorporation when the two kinds of cells were incubated separately. This was also true for LPS cells and PWM cells exposed to radiation.

2.2 The Change in the Function of PWM-activated and LPS-activated Lymphocytes after irradiation *In Vitro*

The incorporation of [^{3}H]TdR in cultures of PWM cells and LPS cells exposed to gamma rays *in vitro* is shown in Table 2 and 3. It can be seen from Table 2 that with increasing radiation dose, the incorporation of [^{3}H]TdR in both PWM cells and LPS cells decreased significantly. When PWM cells and LPS cells were exposed to 10 Gy gamma rays, the decrease was already significant. Thus, in future experiments, we will use 10 Gy gamma-rays.

Table 2 Incorporation of [^{3}H]TdR in PWM-Activated and LPS-Activated Lymphocytes after ^{60}Co γ Irradiation (cpm/5×10^5 cells) (mean ±SE)

	Control	10Gy	20Gy	40Gy
PWM cells	3932±1040	1438±420	1421±419	933±320
LPS cells	930±209	642±121	565±82	538±65

n = 4.

The data in Table 3 show that, when PWM cells and LPS cells were co-cultured, incorporation of [^{3}H]TdR was greater than the total incorporation in PWM cells and LPS

cells when they were incubated separately ($P < 0.01$). This demonstrated that the two kinds of cells had a synergistic effect.

Table 3 Incorporation of [^{3}H]TdR in PWM-Activated Cells Co-Cultured with LPS-Activated Cells before and after Exposure to 10 Gy γ Rays(cpm/5×10^4 cells)(mean ±SE)

	LPS Cells+ PWM Cells	LPS Cells+ PWM Cells after Exposure	LPS Cells after Exposure+ PWM Cells	LPS Cells after Exposure+ PWM Cells after Exposure
Coculture	10442±1265	2350±278	6973±646	1276±96
	LPS Cells	PWM Cells	LPS Cells after Exposure	PWM Cells after Exposure
Single culture	798±156	4192±682	633±85	1301±322
	LPS Cells+ PWM Cells	LPS Cells+ PWM Cells after Exposure	LPS Cells after Exposure+ PWM Cells	LPS Cells after Exposure+ PWM Cells after Exposure
Sum of two single cultures	4990±621	2100±358	4826±647	1936±326

n = 6

when the PWM cells or LPS cells exposed to ^{60}Co gamma rays *in vitro* were co-cultured with normal cells, they incorporated much less [^{3}H]TdR. The incorporation of [^{3}H]TdR in LPS cells + PWM cells after irradiation was the same as the total incorporation in LPS cells and irradiated PWM cells cultured separately; i. e., the synergistic effect disappeared when PWM cells were irradiated. However, the incorporation in co-culture of irradiated LPS cells and PWM cells was still greater than the sum of the incorporation in irradiated LPS cells and PWM cells ($P < 0.05$). The results of these experiments demonstrated that PWM cells played the main role in the synergistic effect and seemed to be more radiosensitive than LPS cells.

When irradiated PWM cells and irradiated LPS cells were co- cultured, the incorporation of [^{3}H]TdR was suppressed significantly and was the same as that found in the non-irradiated LPS cells co-cultured with irradiated PWM cells ($P > 0.05$), lower than that in non-irradiated PWM cells co-cultured with irradiated LPS cells($P < 0.05$). This demonstrated that PWM cells played the main role in the synergistic effect. When irradiated PWM cells and irradiated LPS cells were co-cultured, they incorporated less [^{3}H]TdR than did the two kinds of cells incubated separately.

2.3 The Change in the Function of PWM-activated and LPS-activated Lymphocytes from Patients after Therapy with ^{60}Co Irradiation

The data in Table 4 indicate that the incorporation in LPS celle of the cancer patients

approximated that of normal individuals. When the LPS cells from the patients and PWM cells from normal individuals were co-cultured, the incorporation of [^{3}H]TdR in was not reduced, and was still greater than the sum of incorporation in the two kinds of cells incubated separately. The incorporation in PWM cells of the patients was reduced as compared with that in cells from normal individuals ($P < 0.001$). When PWM cells from the patients and LPS cells from the normal individuals were co-cultured, the incorporation was reduced as compared with that in the co-cultures of the two kinds of cells from normal individuals ($P < 0.001$), although the incorporation was still greater than the total incorporation in the two kinds of cells incubated separately; the differences were not significant ($P > 0.05$). When PWM cells from patients and LPS cells from patients were co-cultured, the incorporation decreased and even was less than the sum of the incorporation in the two kinds of cells incubated separately.

Table 4 Incorporation of [^{3}H]TdR in PWM-Activated Cells Co-Cultured with LPS-Activated Cells from Patients after Radiotherapy and from Normal Individuals (cpm/5×10^5 cells) (mean ±SE)

	Normal LPS Cells + Normal PWM Cells	Normal LPS Cells + PWM Cells from Patients	LPS Cells from Patients + Normal PWM Cells	LPS Cells from Patients + PWM Cells from Patients
Co-culture	11326±1860	4720±1202	10,022±1941	1886±261
	Normal LPS Cells	Normal PWM Cells	LPS Cells from Patients	PWM Cells from Patients
Single culture	1198±99	5533±1453	987±85	2127±372
	Normal LPS Cells + Normal PWM Cells	Normal LPS Cells + PWM Cells from Patients	LPS Cells from Patients + Normal PWM Cells	LPS Cells from Patients + PWM Cells from Patients
Sum of two single cultures	6732±1448	3276±345	6521±1413	3115±344

n = 6

3 DISCUSSION

The [^{3}H]TdR and [^{14}C]UR incorporation methods were adopted to determine the ability of cells to synthesize DNA and RNA, respectively, during lymphocyte transformation.

To study the effect of radiation on the function of T helper cells-B cells, lymphocytes from the same individual were cultured in three combinations with LPS cells + control cells

as the basic model. When control cells were replaced by PWM cells, the LPS cells were stimulated by PWM cells. Furthermore, when they were replaced by γ-irradiated PWM cells the effect of gamma-rays on PWM cells was demonstrated. By comparing the incorporation of [^{3}H]TdR and [^{14}C]UR in PWM cells and LPS cells incubated together with that in PWM cells and LPS cells incubated separately, it showed that the two kinds of cells had a synergistic effect when cultured together.

PWM and LPS are mitogens that induce lymphocyte transformation. In our experiments the incorporation of radioactive compounds was used to determine transformation in PWM-activated cells and LPS-activated cells, the relationship of these two kinds of cells, and their radiosensitivities. It was found that PWM-activated cells were able to enhance the stimulating effect of LPS on B lymphocytes. The stimulating effect of PWM-activated cells became much weaker after the cells were exposed to 40 Gy gamma rays. Because PWM-activated cells include T helper cells and B cells, they are different from PHA-activated OKT$_4$ cells. It was also found that when PWM-activated cells and LPS-activated cells were co-cultured in LPS culture medium, the incorporation of [^{3}H]TdR and [^{14}C]UR was much higher than when the two kinds of cells were incubated separately. This also demonstrated that these two kinds of cells had synergistic effect.

In further experiments the PWM cells and LPS cells were obtained by incubating lymphocytes in PWM and LPS culture medium, respectively. The incorporation of [^{3}H]TdR was greater when the two kinds of cells from the same individual were co-cultured than when they were incubated separately. This demonstrated that they had a synergistic effect. When either PWM or LPS cells were irradiated with 10 Gy gamma rays, the incorporation in co-culture was reduced significantly, and the synergistic effect disappeared. When PWM cells were irradiated, the incorporation in the irradiated PWM cells or irradiated PWM cells co-cultured with LPS cells was suppressed more obviously. This is because PWM-activated cells consist of T helper cells and B cells, which cooperate with each other. When both kinds of cells were irradiated, the incorporation in co-cultured cells was suppressed more significantly than that in the two kinds of cells incubated separately. Further experiments are needed to explain this result.

In the experiments on cells from patients treated with irradiation, the product of the area of the radiation field and radiation dose was used as the unit of exposure (cm^2Gy). Six patients were irradiated with $1.6\sim2.6\times10^4$ cm^2Gy ^{60}Co gamma rays and then PWM cells and LPS cells from patients and healthy persons were co-cultured. The results showed

that the incorporation of [^{3}H]TdR in LPS cells of the patients and normal individuals was nearly the same. When LPS cells from the patients and PWM cells from healthy persons were co-cultured, the incorporation of [^{3}H]TdR was also nearly the same as when the two kinds of cells were taken from healthy individuals. This demonstrated that LPS cells were apparently not damaged. It should be pointed out, however, that the incorporation of [^{3}H] TdR in PWM cells from the patients was reduced significantly and their stimulating effect on normal LPS cells disappeared. Therefore, it could be concluded that PWM cells were seriously damaged after irradiation, which was the further evidence that PWM cells are more radiosensitive than the subpopulation activated by LPS.

Some authors have reported that LPS can induce transformation of B lymphocytes[23,27]. We confirmed this in our previous experiments using isotope incorporation[28]. Other authors [16,19]have reported that PWM could induce T helper cells that stimulate B cells to secrete immunoglobulin and production of immunoglobulin by B lymphocytes activated by PWM. Both $T_4Ia^+$and T_4Ia^- subpopulations can stimulate B lymphocytes[30]. The experiments described above demonstrated the synergistic effect of PWM cells and LPS cells, i. e. , B lymphocytes activated by LPS were subjected to stimulation by PWM, indicating that PWM cells had the function of T helper cells.

When cells were co-cultured, incorporation was influenced when one kind of cells was irradiated. Of the two kinds of cells, the influence was greater in PWM cells was irradiated. The LPS cells in irradiated patients were less damaged, but PWM cells were suppressed significantly and their stimulating effect on B lymphocytes disappeared. This showed that T helper cells played a more important role in synergistic effect. The PWM cells were found to be more radiosensitive than LPS lymphocytes, both in the present study and in our previous studies[8].

Chrest et al. found that B cells from non-responders functioned normally when co-cultured with allogeneic responder T helper cells, whereas T helper cells from non-responders were functionally deficit in activating Ig secretion by B cells of responder[16].

This paper demonstrates that the effect of radiation on the immune response in healthy individuals or lymphocytes was identical to the effect on non-responders : the T helper cells are functionally deficient in activating B cells in responders or non-responders.

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