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Eradication of Tumour Cells by Successive Injections of Allogeneic Immune and Hyperimmune Peritoneal Cells in a Murine Lymphoma System*

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Abstract—*Allogeneic C57BL immune and hyperimmune (vs SL2) peritoneal cells are used for eradication of DBA/2 derived SL2 lymphoma cells injected into the peritoneal cavity of DBA/2 mice.*

SL2 bearing DBA/2 mice are treated with 3, 5, or 8 successive i.p. injections of 2×10^6 allogeneic C57BL immune or hyperimmune peritoneal macrophages (contaminated with lymphocytes). 2×10^7 SL2 cells could be eradicated successfully with five injections of immune or three injections of hyperimmune C57BL peritoneal cells. This is a ten-fold improvement compared to the result obtained with single injections of immune or hyperimmune peritoneal cells.

The number of injections with immune or hyperimmune cells, time intervals between the injections and the total length of the period of the therapy was important for the result of the therapy.

INTRODUCTION

ALLOGENEIC immune and hyperimmune peritoneal exudate cells, comprising about equal numbers of macrophages and lymphocytes, are very effective tools in immunotherapy of ascitic lymphoma cells injected into the syngeneic host [1–3]. With one injection of 2×10^6 immune or hyperimmune peritoneal macrophages, contaminated with an approximately equal number of lymphocytes, 2×10^6 SL2 cells can be eradicated.

In this paper, experiments are described in which SL2 bearing DBA/2 mice are treated with successive i.p. injections of allogeneic immune or hyperimmune peritoneal cells. With this technique, even 2×10^7 SL2 cells can be eradicated. The importance of this result is clear if this number of cells is compared to the maximum number of cells that can be obtained from a transplantation mouse which is 5×10^8 SL2 cells. Furthermore, it has always been stressed that with immunotherapy, only a very small number of tumour cells, no more than 10^3–10^5 cells, can be eradicated [4–6].

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MATERIAL AND METHODS

Mice

Pure bred DBA/2, C57BL and CBA mice, 6–10 weeks old were used. DBA/2 mice were used as: (a) a source of DBA/2 derived SL2 lymphoma cells; (b) SL2 bearing mice to be cured; and (c) as a source of DBA/2 liver cells. C57BL mice were used as a source of allogeneic non-immune, immune or hyperimmune peritoneal, spleen and lymphnode cells. CBA mice

were used as a source of CBA derived TLC5 lymphoma cells.

Lymphomas

The DBA/2 derived lymphoma SL2, and the CBA derived lymphoma TLC5 were maintained by weekly i.p. passage.

Both lymphomas were gifts from Dr. P. Alexander.

Immunization

C57BL mice were immunized by one or four i.p. injections of 10^7 SL2, 10^7 TLC5 or 10^7–10^8 DBA/2 liver cells.

Immunization schedules

(a) C57BL mice were immunized by one i.p. injection of 10^7 SL2, 10^7 TLC5 or 10^7–10^8 DBA/2 liver cells. Peritoneal cells were harvested 10 days later. This is the optimal time for collection of allogeneic immune peritoneal cells [1, 2]. (b) C57BL mice were hyperimmunized by four injections at days 0, 9, 18 and 27. The peritoneal cells were collected 5 days after the last immunization. (This is the optimal time for collection of allogeneic hyperimmune peritoneal cells [3]).

Collection of peritoneal cells

Peritoneal cells (non-immune, immune or hyperimmune) were collected by washing the peritoneal cavity with 5 ml Fischer's medium. Cells were centrifuged and resuspended in Fischer's medium [7].

Cells used for therapy

(a) 2×10^6 immune or hyperimmune (vs. SL2) allogeneic C57BL peritoneal macrophages/ml. The macrophages were contaminated with an approximately equal number of lymphocytes [1, 2].

Cells used as controls

(a) 2×10^6 immune or hyperimmune (vs TLC5) allogeneic C57BL peritoneal macrophages/ml. Macrophages were contaminated with lymphocytes [1, 2]. (b) 2×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL peritoneal lymphocytes/ml. These lymphocytes were freed of macrophages by twice exposing the exudate to glass for 30 min at 37°C. Lymphocytes were contaminated with less than 7% macrophages as counted in a haemocytometer. (c) 2×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL peritoneal macrophages/ml. These macrophages were freed of lymphocytes by injecting 0·3 ml anti lymphocyte serum (ALS)† i.p. into immune or hyperimmune C57BL mice 45 min before collection of the peritoneal cells. Macrophages were contaminated with less than 8% lymphocytes as counted in a haemocytometer. (d) 2×10^6 non-immune (normal) C57BL allogeneic peritoneal macrophages/ml. Macrophages were contaminated with lymphocytes. (e) 2×10^6 non-immune allogeneic C57BL peritoneal macrophages/ml, freed of lymphocytes (see also c). Macrophages were contaminated with less than 7% lymphocytes as counted in a haemocytometer. (f) 2×10^6 non-immune allogeneic C57BL peritoneal lymphocytes/ml, freed of macrophages (see also b). Lymphocytes were contaminated with less than 5% macrophages. (g) 2×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL lymph node cells/ml. Lymphoid cells were obtained from the lymph-nodi mandibulares. Lymph nodes were cut in Fischer's medium. (h) 2×10^6 non-immune allogeneic C57BL lymph node cells/ml (see also g). (i) 4×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL lymphoid cells of the spleen/ml. Spleen cells were obtained by rinsing out the spleen by injecting 5 ml of Fischer's medium. (j) 4×10^6 non-immune allogeneic C57BL lymphoid cells of the spleen/ml. (k) 2×10^6 immune or hyperimmune (vs DBA/2 liver) C57BL peritoneal macrophages/ml. Macrophages were contaminated with lymphocytes.

RESULTS

Therapy with immune peritoneal cells

The results of the first series of experiments are summarized in Table 1. It was shown that treatment of SL2 bearing mice with 5 i.p. injections of immune C57BL peritoneal cells was most effective in eradication of tumour cells. Two out of five mice survived a dose of as much as 2×10^7 SL2 cells i.p. for more than 35 days. Treatment of SL2 bearing mice with 8 injections of allogeneic immune peritoneal cells was less successful than with 3 or 5 injections.

Therapy with hyperimmune peritoneal cells

The effect of treatment of SL2 bearing mice with 3 or 5 i.p. injections of 2×10^6 C57BL hyperimmune (vs SL2) peritoneal macrophages (contaminated with lymphocytes) is shown in Table 2. Two out of five DBA/2 mice treated with 3 i.p. injections of hyperimmune cells

†ALS: ALS was a horse-anti mouse serum. It was a gift from Radiobiological Institute, TNO, Rijswijk, The Netherlands.

Table 1. *Effect of successive* injections with* 10 *days immune (vs SL2) allogeneic C57BL peritoneal cells into SL2 bearing DBA/2 mice*

	Number of survivors at 35 days (real survival time in days in parentheses) after treatment					
No. of SL2 cells	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7	10^8
Treatment with						
3 i.p. injections of immune peritoneal cells†	5/5 (5 > 150)	4/5 (59,3 > 150)	3/5 (3 > 150)	4/5 (4 > 150)	0/5 (10·2 ± 0·2)	0/5 (8·6 ± 1·4)
3 i.p. injections of Fischer's medium only	0/5 (19·0 ± 0·0)	0/5 (18·0 ± 0·0)	0/5 (16·0 ± 0·0)	0/5 (15·0 ± 0·0)	0/5 (9·0 ± 0·0)	0/5 (8·0 ± 1·0)
5 i.p. injections of immune peritoneal cells†	4/5 (35·3 > 150)	3/5 (39, 59, 1 > 150)	2/5 (2 > 150)	3/5 (37, 40, 1 > 150)	2/5 (2 > 150)	0/5 (9·0 ± 0·0)
5 i.p. injections of Fischer's medium only	0/5 (13·0 ± 0·0)	0/5 (10·5 ± 0·5)	0/5 (10·0 ± 0·0)	0/5 (10·0 ± 0·0)	0/5 (9·0 ± 0·0)	0/5 (8·0 ± 0·0)
8 i.p. injections of immune peritoneal cells†	2/5 (35, 51)	1/5 (72)	0/5 (23·6 ± 4·5)	2/5 (38, 1 > 150)	0/5 (14·2 ± 1·2)	Not done
8 i.p. injections of Fischer's medium only	0/5 (14·6 ± 0·8)	0/5 (13·6 ± 0·5)	0/5 (13·6 ± 0·8)	0/5 (11·0 ± 0·0)	0/5 (10·0 ± 0·0)	Not done

*The first injection was given 2 hr after challenge, the following injections were given with time intervals of 24 hr.
†2×10^6 allogeneic immune peritoneal C57BL macrophages contaminated with an approximately equal number of lymphocytes; the immune cells were collected 10 days after immunization.

Table 2. *Effect of successive* i.p. injections with hyperimmune (vs SL2) allogeneic C57BL peritoneal cells into SL2 bearing DBA/2 mice*

	Number of survivors at 35 days (real survival time in days in parentheses) after treatment			
No. of SL2 cells	2×10^5	2×10^6	2×10^7	10^8
Treatment with				
3 i.p. injections of hyperimmune peritoneal cells†	4/5 (38, 44, 2 > 150)	3/5 (42,2 > 150)	2/5 (35,1 > 150)	0/5 (10·4 ± 1·0)
3 i.p. injections of Fischer's medium only	0/5 (11·5 ± 0·5)	0/5 (11·5 ± 0·5)	0/5 (10·0 ± 1·0)	0/5 (10·5 ± 0·5)
5 i.p. injections of hyperimmune peritoneal cells†	4/5 (60,3 > 150)	3/5 (38,2 > 150)	0/5 (12·8 ± 1·6)	Not done
5 i.p. injections of Fischer's medium only	0/5 (14·0 ± 0·0)	0/5 (11·5 ± 0·5)	0/5 (8·5 ± 0·5)	Not done

*The first injection was given 2 hr after challenge, the following injections were given with time-intervals of 24 hr.
†2×10^6 allogeneic peritoneal macrophages from C57BL mice immunized at day 0, 9, 18, 27; the cells were collected 5 days after the last immunization, macrophages were contaminated with an approximately equal number of lymphocytes.

survived a dose of 2×10^7 SL2 cells for more than 35 days. When treated with 5 i.p. injections with hyperimmune peritoneal cells, only a dose of 2×10^6 SL2 cells could be eradicated: three out of five mice survived more than 35 days after challenge.

Therapy with 3 *successive injections with immune or hyperimmune peritoneal cells within* 24 *hr after challenge*

SL2 bearing DBA/2 mice were treated with 3 successive i.p. injections of 2×10^6 allogeneic C57BL immune or hyperimmune peritoneal macrophages (contaminated with lymphocytes) 2, 13, and 24 hr after challenge. The number of survivors at 35 days after challenge decreases considerably (Table 3).

When DBA/2 mice (not injected with SL2 cells) were treated with 3 i.p. injections of 2×10^6 immune C57BL peritoneal macrophages (contaminated with lymphocytes) within 24 hr, 1 out of 5 mice died. However, this was not caused by the allogeneic cells, but was due to proliferating SL2 cells present among the 10 days immune cells [1, 2],

Three i.p. injections hyperimmune peritoneal cells within 24hr caused no harm to the recipients.

Comparison of the 3 *foregoing series of experiments*

Comparison of the 3 foregoing series of experiments (Table 4) shows that treatment with 5 i.p. injections of allogeneic *immune*

*Table 3. Effect of 3 successive i.p. injections of allogeneic immune or hyperimmune (vs SL2) C57BL peritoneal cells into SL2 bearing DBA/2 mice within 24 hr**

	Number of survivors at 35 days (real survival time in days in parentheses) after treatment				
No. of SL2 cells	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7
Treatment with					
3 i.p. injections of immune peritoneal cells†	1/5 (1 > 150)	0/5 (21·6 ± 0·8)	0/5 (22·2 ± 5·2)	0/5 (21·2 ± 2·9)	0/5 (10·6 ± 1·0)
3 i.p. injections of hyperimmune peritoneal cells†	Not done	Not done	4/5 (49, 3 > 150)	0/5 (24·4 ± 3·2)	0/5 (15·4 ± 2·7)
3 i.p. injections of Fischer's medium only	0/5 (15·0 ± 0·0)	0/5 (14·0 ± 1·0)	0/5 (12·5 ± 0·5)	0/5 (11·8 ± 0·4)	0/5 (9·8 ± 0·4)

*The injections were given 2, 12, and 24 hr after challenge with SL2 cells.

†2×10^6 immune or hyperimmune peritoneal C57BL macrophages contaminated with lymphocytes.

Table 4. Comparison of the results of the three foregoing series of experiments as summarized in Tables 1, 2 and 3

	Number of survivors at 35 days after challenge		
No. of SL2 cells	2×10^5	2×10^6	2×10^7
Treatment with			
3 i.p. injections* of immune peritoneal cells†	3/5	4/5	0/5
5 i.p. injections* of immune peritoneal cells†	2/5	3/5	2/5
8 i.p. injections* of immune peritoneal cells†	0/5	2/5	0/5
3 i.p. injections* of hyperimmune peritoneal cells†	4/5	3/5	2/5
5 i.p. injections of hyperimmune peritoneal cells†	4/5	3/5	0/5
3 i.p. injections‡ of immune peritoneal cells† within 24 hr	0/5	0/5	0/5
3 i.p. injections‡ of hyperimmune peritoneal cells† within 24 hr.	4/5	0/5	0/5

*The first injection was given 2 hr after challenge with SL2 cells, the rest of the injection was given with time intervals of 24 hr.

†2×10^6 immune or hyperimmune C57BL peritoneal macrophages contaminated with lymphocytes.

‡The injections were given 2, 13 and 24 hr after challenge.

peritoneal cells, or with 3 i.p. injections of allogeneic *hyperimmune* peritoneal cells gives the best results in eradication of tumour cells. Treatment of SL2 bearing DBA/2 mice with 3 i.p. injections with immune or hyperimmune peritoneal cells within 24 hr after challenge was less successful.

Control cell suspensions

Experiments in which the control suspensions were tested showed that only immune or hyperimmune peritoneal lymphocytes were able to eradiacate 2×10^5 SL2 cells. When DBA/2 mice were challenged with 2×10^5 SL2 cells and treated with 3 successive i.p. injections with 2×10^6 C57BL immune or hyperimmune peritoneal lymphocytes 1 out of 5, and 3 out of 5 mice respectively survive for more than 35 days (Table 5). However, when DBA/2 mice were injected i.p. with 2×10^6 SL2 cells and treated with 3 i.p. injections of immune or hyperimmune peritoneal lymphocytes, none of the mice survived for more than 35 days. Further, only treatment with 3 successive i.p. injections of 4×10^6 C57BL hyperimmune lymphoid cells of the spleen, and 2×10^6 C57BL hyperimmune peritoneal macrophages caused a prolongation of life time of the SL2 bearing DBA/2 mice. With the rest of the control suspensions, only a very slight prolongation of lifetime or no prolongation at all was obtained.

DISCUSSION

It is well established that resistance to allograft murine tumours can be transferred by immune or hyperimmune peritoneal cell suspensions [2, 3, 8–11]. Transfer of immunity by immune [12–14] or hyperimmune lymphocytes [3] and hyperimmune spleen cells [3, 4] has been reported. It has been shown also that passive transfer of allogeneic immuno-competent lymphoid cells under certain conditions may hold in check experimental tumours temporarily and sometimes even destroy the tumour completely [15–18].

The experiments described in this paper show that treatment of SL2 bearing DBA/2 mice with successive i.p. injections of allogeneic immune or hyperimmune peritoneal cells is very successful (Tables 1, 2).

Table 5. *Effect of* 3 *successive* i.p. injections of C57BL control cell suspensions into DBA/2 mice injected i.p. with* 2×10^5 *SL* 2† *cells*

Cell suspension	Number of survivors at 35 days (real survival time in parentheses) after treatment		
	Non-immune	Immune	Hyperimmune
2×10^6 peritoneal lymphocytes	0/5 (12·0 ± 1·6)	1/5 (42)	3/5 (36, 37, 1 > 150)
2×10^6 peritoneal macrophages	0/5 (13·2 ± 0·4)	0/5 (15·0 ± 0·6)	0/5 (17·6 ± 2·6)
2×10^6 lymph node cells	0/5 (12·6 ± 0·5)	0/5 (12·8 ± 0·8)	0/5 (13·0 ± 0·0
4×10^6 spleen cells	0/5 (12·8 ± 0·4)	0/5 (15·4 ± 1·1)	0/5 (27·0 ± 3·3)
2×10^6 peritoneal macrophages contaminated with lymphocytes	—	0/5‡(12·2 ± 0·7)	0/5‡(15·4 ± 2·2)
2×10^6 peritoneal macrophages contaminated with lymphocytes	—	0/5§(12·2 ± 0·7)	0/5§(11·2 ± 0·4)
2×10^6 peritoneal macrophages contaminated with lymphocytes	0/5 (12·2 ± 0·5)	—	—

*injection were given 2, 24 and 48 hr after injection SL2 cells.

†controls, injected with 2×10^5 SL2 cells only died after 12·0 ± 0·0 days.

‡immune, or hyperimmune vs CBA derived TLC5 lymphoma.

§immune, or hyperimmune vs DBA/2 liver cells.

The number of SL2 cells that could be eradicated, however, did not correlate simply with the number of injections with allogeneic immune or hyperimmune peritoneal cells. Treatment of SL2 bearing DBA/2 mice with 8 i.p. injections of allogeneic immune peritoneal cells reduced significantly the number of survivors at 35 days after challenge.

This reduction possibly might be due to a sensitization reaction of the DBA/2 host caused by the successive administration of allogeneic cells [12] resulting in rejection of the immune cells. Rejection of these allogeneic immune cells may lead to a "free" proliferation of SL2 lymphoma cells which might still be present in the immune peritoneal cell suspension obtained from mice immunized 10 days earlier [1, 2], and which might finally kill the mouse. Den Otter *et al.* [19] were able to show that only 1 SL2 cell i.p. is sufficient to kill a DBA/2 mouse within 26 days.

Development of a severe Graft vs Host reaction eventually resulting in early death of the test animals, as described by Woodruff *et al.* [17, 18] was not noticed as all mice which died within 35 days after challenge had a fully developed tumour.

A significant reduction of the number of survivors at 35 days after challenge was also shown when SL2 bearing DBA/2 mice were treated with 3 i.p. injections of immune or hyperimmune peritoneal cells within 24 hr after challenge. However, treatment of DBA/2 mice (not injected with SL2 cells) with 3 i.p. injections of immune or hyperimmune peritoneal cells within 24 hr caused no harm to the recipients. Further investigation on this subject will be necessary.

In control experiments, it was shown that only treatment of DBA/2 mice, injected i.p. with 2×10^5 SL2 cells by 3 successive i.p. injections of 2×10^6 *immune* or *hyperimmune* C57BL peritoneal lymphocytes (contaminated with 7 % macrophages) resulted in survival of 1 out of 5, and 3 out of 5 mice respectively, for more than 35 days after challenge (Table 5). However, when DBA/2 mice were challenged with 2×10^6 SL2 cells, there were no survivors at 35 days. Further, only treatment with hyperimmune spleen cells resulted in a significant prolongation of life-time, but there were also no survivors at 35 days.

These results approve earlier findings [1–3]: (a) optimal therapeutic effects in this allogeneic system are obtained when a suspension of peritoneal macrophages contaminated with lymphocytes is used, and (b) the reaction described in this paper may be regarded as immunologically specific as treatment with peritoneal cells from C57BL mice immunized or hyperimmunized against CBA derived TLC5 lymphoma, or against DBA/2 liver cells did not result in significant prolongation of life.

REFERENCES

1. H. F. J. Dullens, and W. Den Otter, Eradication of lymphoma cells with allogeneic immune peritoneal cells. *Experientia* (*Basel*) **29,** 479 (1973).

2. H. F. J. Dullens and W. Den Otter, Therapy with allogeneic immune peritoneal cells. *Cancer Res.* **34,** 1726 (1974).
3. H. F. J. Dullens, F. J. Kingma and W. Den Otter, Immunotherapy with allogeneic hyperimmune peritoneal cells in a murine lymphoma system. *Europ. J. Cancer* **10,** 41 (1974).
4. P. Alexander, Prospects for immunotherapy of cancer: experience in experimental systems. *Brit. med. J.* **4,** 484 (1970).
5. P. Alexander, D. I. Connell and Z. B. Mikulska, Treatment of a murine leukemia with spleen cells or sera from allogeneic mice immunized against the tumour. *Cancer Res.* **26,** 1508 (1966).
6. G. Mathé, Immunological treatment of leukemias. *Brit. med. J.* **4,** 487 (1970).
7. G. A. Fischer, Studies of the culture of leukemia *in vitro*. *Ann. N.Y. Acad. Sci.* **76,** 673 (1958).
8. D. B. Amos, In *Mechanism of Cell and Tissue Damage Production by Immune Reactions. II. International Symposium on Immunopathology.* (Edited by P. Grabar and P. Miescher), Schwabe, Basel (1962).
9. P. Baker, R. S. Weiser, J. Jutila, C. E. Evans and R. J. Blandau, Mechanisms of tumor homograft rejection: the behavior of sarcoma I ascites tumor in the A/Jax and C57BL/6K mouse. *Ann. N.Y. Acad. Sci.* **101,** 46 (1962).
10. B. Bennett, Specific suppression of tumour growth by isolated peritoneal macrophages from immunized mice. *J. Immunol.* **95,** 656 (1965).
11. W. E. Hoy and D. S. Nelson, Studies on Cytophylic antibodies. V. Alloantibodies cytophylic for mouse macrophages. *Aust. J. exp. Biol. med. Sci.* **47,** 525 (1969).
12. H. Borberg, H. F. Oettgen, K. Choudry and E. J. Beattie, Jr., Inhibition of established transplants of chemically induced sarcomas in syngeneic mice by lymphocytes from immunized donors. *Int. J. Cancer* **10,** 539 (1972).
13. E. J. Delorme and P. Alexander, Treatment of primary fibrosarcoma in the rat with immune lymphocytes. *Lancet* **18,** 117 (1964).
14. B. Klein, H. O. Sjögren, E. Klein and K. E. Hellström, Demonstration of resistance against methylcholantrene induced sarcomas in the primary autochtonous host. *Cancer Res.* **20,** 1561 (1960).
15. L. Ellman, D. H. Katz, I. Green, W. E. Paul and B. Benacerraf, Mechanisms involved in the antileukemic effect of immunocompetent allogeneic lymphoid cell transfer. *Cancer Res.* **32,** 141 (1972).
16. D. H. Katz, L. Ellman, W. E. Paul. I. Green and B. Benacerraf, Reistance of guinea pigs to leukemia following transfer of immunocompetent allogeneic lymphoid cells. *Cancer Res.* **32,** 133 (1972).
17. M. F. A. Woodruff and M. O. Symes, The use of immunologically competent cells in the treatment of cancer. Experiments with a transplantable mouse tumour. *Brit. J. Cancer* **16,** 707 (1962).
18. M. F. A. Woodruff and J. L. Boak, Inhibitory effect of pre-immunized CBA spleen cells on transplants of L-strain mouse mammary carcinoma in (CBA × A) F_1 hybrid recipients. *Brit. J. Cancer* **19,** 411 (1965).
19. W. Den Otter, E. A. Runhaar, C. A. Ruitenbeek and H. F. J. Dullens, Site-dependent differences in rejection of tumour cells with and without preimmunization. *Europ. J. Immunol.* **4,** 444 (1974).