The Role of Complete Freund's Adjuvant in the Immunoprotection of L_2C Guinea Pig Leukemia (35942)

LEONARD EILMAN AND IRA GREEN

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

The immunotherapy of human leukemia (1–3) by active immunization with tumor cells is currently being intensively investigated. A crucial question for these studies is the determination of the optimal node of immunization with tumor cells or tumor cell extracts. Although the use of complete Freund's adjuvant (CFA) is a classic means of augmenting the immune response, especially the cellular immune response (4), the role of CFA in tumor immunology has not been systematically studied. It is of great importance to learn whether the immunization of patients with tumor cells or tumor cell extracts in CFA would be superior to other forms of immunization. In the present report, a well-studied guinea pig leukemia model has been utilized to directly compare the immunoprotection achieved by immunization with irradiated tumor cells either administered in aqueous medium or in CFA.

Guinea pig leukemia L_2C, a primarily lymphatic leukemia, arose spontaneously in an inbred strain 2 guinea pig (5). This leukemia has been serially transplanted in strain 2 guinea pigs for over 15 years; it is not readily transplantable to adult strain 13 or random bred guinea pigs (6). A viral etiology for leukemia L_2C has been proposed (7–9), but this has not been generally confirmed (10–12).

In recent studies (13), we have demonstrated the presence of a tumor specific transplantation antigen (TSTA) on L_2C cells by immunoprotection tests. These studies demonstrated that immunization of strain 2 guinea pigs with large numbers of irradiated L_2C cells injected either in aqueous medium, or emulsified in CFA and injected in the foot pads protects almost every animal against lethal leukemic challenge administered 12 days later. In the present study, we wished to determine what form of immunization is superior. Therefore, immunization protection tests were performed under conditions expected to produce a greater challenge to the animal's ability to reject the tumor: immunized animals were given a lethal challenge at shorter intervals after immunization and/or were challenged with a very large leukemic inoculum. Using these more stressful conditions, a definite advantage to the use of CFA has been demonstrated.

Materials and Methods. Experimental animals. Inbred strain 2 guinea pigs were obtained from the Rodent Production Section, National Institutes of Health.

Experimental tumors. Our initial supply of L_2C leukemia was kindly provided by Dr. L. S. Kaplow, Yale University School of Medicine, New Haven, Conn. The leukemia was passaged three times in our strain 2 colony and a large pool of L_2C cells was obtained from the blood of leukemic animals after sedimentation of the red cells with 1% gelatin in saline. The L_2C cells were washed three times in Eagle's medium and preserved in 10% dimethylsulfoxide at −70°. These cells were used for both immunization and challenge. Trypan blue exclusion indicated that 70–90 % of the L_2C cells from different aliquots stored in this manner were viable after re-thawing. The number of these viable cells necessary to produce death in an untreated strain 2 animal (see below) was similar to the number of cells required when obtained directly from an animal with leukemia.

Immunization protection tests. Adult strain 2 guinea pigs were immunized with L_2C cells which had been irradiated with 600 R. In each experiment, one group of animals was

TABLE I. 90 Day Survival Following Intradermal Challenge With 3×10^5 L_2C Cells.

Interval (days) between immunization and leukemic challenge	Immunization: 50×10^6 irradiated L_2C cells				Controls	
	In aqueous medium, injected intradermally		In complete Freund's adjuvant, injected in foot pads			
2	1/4[a]	(45, 48, 55)[b]	3/4	(53)	0/2	(21, 22)
3 a[d]	1/5	(19, 38, 42, 50)	3/5	(49, 53)	0/1	(21)
b[d]	1/5	(19, 24, 44, 56)	4/5	(42)	0/1	(21)
Cumulative survival	3/14[c]		10/14[c]			

[a] Ninety day survivors/total number of animals at risk.

[b] Interval in days between leukemic challenge and death of animal.

[c] $p < .05$.

[d] a and b represent the same experiment performed at two different times.

immunized by the intradermal injection into multiple sites of 50×10^6 L_2C cells contained in 1 ml of physiologic buffered (PO_4) saline, pH 7.6 (aqueous immunization). The other group of strain 2 animals were immunized with an emulsion prepared by mixing L_2C cells contained in physiologic saline with an equal volume of CFA containing 0.5 mg/ml of *Mycobacterium butyricum* (Difco Laboratories, Detroit, Michigan). 0.4 ml of the emulsion containing 50×10^6 L_2C cells was distributed among the four foot pads. A small number of untreated strain 2 animals served as a control for each experiment.

Leukemic challenge consisted of the intradermal injection of 3×10^5 or 3×10^6 L_2C cells contained in 0.1 ml of physiologic saline. In previous experiments (13), 1×10^5 L_2C cells was found to be uniformly lethal for strain 2 guinea pigs following intradermal injection; thus, following challenge with 3×10^5 L_2C cells, 36 control strain 2 guinea pigs had a mean survival of 22 days with a range of survival of 16 to 28 days. Challenge with 3×10^6 L_2C cells uniformly led to an accelerated time of death of control strain 2 animals with a mean survival of 16 days. In our previous study, animals surviving 90 days following leukemic challenge appeared to be permanently protected (13); in the present experiment, animals surviving 90 days were therefore considered to be permanently protected.

Results. Survival following challenge with 3×10^5 L_2C Cells. In earlier experiments (13), we found little difference between aqueous and CFA immunization when leukemic challenge with 3×10^5 cells was administered 12 days after immunization: 11/12 aqueous immunized animals and 23/24 CFA immunized animals were permanently protected. In the present experiments, immunization 6 days before leukemic challenge also failed to show a difference between CFA and aqueous immunization: the four animals in each group were alive at 90 days. However, when the leukemic challenge was administered only 2 or 3 days following immunization, CFA immunization was clearly superior (Table I). It would appear that the CFA immunization accelerates the development of a high degree of tumor immunity. Although aqueous immunization led to a prolongation of life in many cases, a sufficiently high level of tumor immunity did not develop early enough to actually eradicate the tumor.

Survival following challenge with 3×10^6 L_2C cells. Following this greater leukemic challenge, the superiority of CFA immunization is apparent at all time periods (Table II), although aqueous immunization enables predictable rejection of 3×10^5 L_2C cells 12 days after immunization, this form of immunization does not appear to induce sufficient tumor immunity to reject a tenfold larger leukemic challenge. Moreover, with two exceptions, animals immunized with tumor cells in CFA, 5 and 12 days before challenge with 3×10^6 L_2C cells, manifested erythema and induration at the site of intradermal challenge appearing 24 hr later. This reaction has

TABLE II. 90 Day Survival Following Intradermal Challenge with 3×10^6 L_2C Cells.

Interval (days) between immunization and leukemic challenge	Immunization: 50×10^6 irradiated L_2C cells				Controls	
	In aqueous medium, injected intradermally		In complete Freund's adjuvant, injected			
3	0/5[a]	(23, 27, 33, 41, 43)[b]	2/5	(36, 37, 41)	0/1	(16)
5	1/5	(18, 33, 41, 47)	4/5	(32)	0/1	(14)
12	1/5	(20, 34, 39, 40)	5/5		0/1	(15)
Cumulative survival at all time periods	2/15[c]		11/15[c]			

[a] Ninety day survivors/total number of animals at risk.
[b] Interval in days between leukemic challenge and death of animal.
[c] $p < .01$.

been shown to be a delayed hypersensitivity reaction to the L_2 cells in immunized animals (13). No such skin reaction was evident in any of the aqueous immunized animals.

Discussion. The present studies demonstrate a clear advantage to the use of CFA over aqueous medium for the immunization of strain 2 guinea pigs against leukemia L_2C. Immunization with L_2C cells emulsified in CFA allows for both the more rapid development of tumor immunity, and the development of a sufficiently high level of immunity to reject a very large leukemic challenge.

We are aware of only one other study in which the immunoprotective effect of immunization of tumor material in aqueous medium and CFA was directly compared (14). In this study, Fink *et al.* (14) also found a marked advantage of the use of CFA: BALB/c mice immunized with a homogenate of a chemically induced syngeneic fibrosarcoma incorporated into CFA demonstrated considerably greater immunoprotection than animals immunized with the homogenate alone. Although the mode of action of mycobacterial adjuvants remains poorly understood, they are classic tools in immunology to augment the immune response, especially the cellular immune response (4). Thus, the beneficial effects of administering tumor cells or tumor cell extracts in CFA are not unexpected.

Recently, Zbar and associates (15) have reported induction of tumor immunity to a diethylnitrosamine-induced hepatoma in the strain 2 guinea pig by the intradermal inoculation of syngeneic living tumor cells mixed with living BCG. An inflammatory reaction to the BCG occurs approximately 1 week later, and, as a result, there is a failure of progressive local tumor growth. These animals subsequently are able to specifically reject lethal rechallenges with this hepatoma, but fail to reject a different line of carcinogen-induced hepatoma. These investigators have also demonstrated that for the development of optimal tumor immunity, direct contact between tumor cells and BCG is required (16).

In contrast to the results with strain 2 hepatoma, immunization of strain 2 guinea pigs with living L_2C cells and living BCG failed to protect the animals for leukemia (Berton Zbar, personal communication). We attribute this failure to the very rapid rate of proliferation of the L_2C cells and the tendency for early dissemination of these leukemia cells. Thus, the leukemia was probably widely disseminated by the time the inflammatory response to the BCG had developed. However, our experience suggests that intradermal immunization with irradiated (inactivated) L_2C cells and BCG would be another effective means of inducing tumor immunity to L_2C cells.

A distinction must be made between administering tumor cells or tumor cell extracts in mycobacterial adjuvants and the use of mycobacterial adjuvants alone as a nonspecific stimulator of the immune system. The latter procedure has led to prolongation of survival in some experimental tumor systems,

but not in others (13, 15, 17–19). Mathé *et al.* (3) have recently treated patients with acute leukemia in remission with BCG, inactivated, pooled tumor cells, or a combination of the two regimens; it is noteworthy that the tumor cells and BCG were not administered together. Although the results of the latter study are encouraging, the observation that BCG treatment alone was as effective as the tumor cell inoculum regimes suggests that the antileukemic response was based on a nonspecific phenomena rather than on a specific immune response to TSTA present on the leukemic cells.

The immunization of a large number of patients who have solid malignancy with autologous tumor extract in CFA has been studied by Graham and Graham (20) with disappointing results. However, the patients all had advanced disease. Recently, Hughes *et al.* (21) immunized 20 patients with advanced solid tumors with autologous tumor extract incorporated serially into CFA, incomplete Freund's adjuvant, and typhoid and pertussis vaccine. Although the clinical response was not striking, development of a cellular immune response to the tumor was demonstrated in many cases. The procedure proved safe and in no case did a patient develop a local abscess following the first injection of CFA. Subcutaneous abscesses which developed following repeated injections of CFA were easily managed by excision or drainage.

Since recent attempts at immunotherapy of human leukemia offer some encouragement (1–3), further attempts at vigorous immunization of patients with leukemia with autologous or pooled tumor cells or extracts during periods of remission seem justifiable. We feel that full advantage should be taken of the powerful adjuvant effect of CFA and BCG, and that in future immunotherapy trials, tumor cells or extracts should be mixed directly with the mycobacterial adjuvant prior to administration.

Summary. Leukemia L_2C is a syngeneic guinea pig leukemia which grows progressively in strain 2 guinea pigs. In previous studies, we have demonstrated that immunization of strain 2 guinea pigs with irradiated L_2C cells in aqueous medium or in complete Freund's adjuvant 12 days before lethal leukemic challenge successfully protected almost every animal against leukemia. In the present study, immunization with irradiated L_2C cells in aqueous medium and complete Freund's adjuvant was directly compared using immunization period of shorter duration and challenges with higher numbers of leukemic cells. Under these conditions a distinct advantage to the use of complete Freund's adjuvant was observed with the more rapid development of high levels of tumor immunity. These studies with guinea pig leukemia suggest that in immunotherapy trials of human leukemia, tumor cells or extracts should be administered directly in mycobacterial adjuvants.

The authors acknowledge the expert technical assistance of Mrs. Clara Horton.

1. Skurkovich, S. V., Kisljak, W. S., Makhonova, L. A., and Begunenko, S. A., Nature **223**, 509 (1969).

2. Skurkovich, S. V., Makhonova, L. A., Reznichenko, F. M., and Cherovonskiy, G. I., Blood **33**, 186 (1969).

3. Mathé, G., Amiel, L. J., Schwarzenberg, L., Schneider, M., Cottan, A., Schlumberger, J. R., Hayat, M., and deVassal, F., Lancet **1**, 697 (1969).

4. Freund, J., Advan. Tuberc. Res. **1**, 130 (1956).

5. Congdon, C. C., and Lorenz, E., Amer. J. Pathol. **30**, 337 (1954).

6. Nadel, E. M., J. Nat. Cancer Inst. **19**, 351 (1957).

7. Opler, S. R., J. Nat. Cancer Inst. **38**, 797 (1967).

8. Nadel, E., Banfield, W., Burstein, S., and Tousimia, A. J., J. Nat. Cancer Inst. **38**, 979 (1967).

9. Jungeblut, C. W., and Opler, S. R., Amer. J. Pathol. **51**, 1153 (1967).

10. Wepsic, H. T., Zbar, B., Whang-Peng, J., Borsos, T., and Rappl, H. J., J. Nat. Cancer Inst. **45**, 99 (1970).

11. Gross, L., Dreyfuss, Y., Ehrenreich, T., and Moore, L. A., Acta Haematol. **43**, 193 (1970).

12. Sarma, P. S., Uterhorst, P. T., Zeve, V., Wepsic, H. T., Whang-Peng, J., Zbar, B., and Hueber, R. J., Proc. Int. Symp. Comp. Leukemia Rec., 4th, Cherry Hill, NJ (1969).

13. Ellman, L., and Green, I., Cancer **28**, 647 (1971).

14. Fink, M. A., Snell, G. D., and Kelton, D., Cancer Res. **13**, 666 (1953).

15. Zbar, B., Bernstein, I., Tanaka, T., and Rapp, H. J., Science **170**, 1217 (1970).

16. Zbar, B., and Tanaka, T., Science **172**, 271 (1971).

17. Old, L. J., Benacerraf, B., Clark, D. A., Carswell, E. A., and Stockert, E., Cancer Res. **21**, 1281 (1961).

18. Weiss, D. W., Bonhag, R. S., and DeOme, K. B., Nature (London) **190**, 889 (1961).

19. Weiss, D. W., Bonhag, R. S., and Leslie, P., J. Exp. Med. **124**, 1039 (1966).

20. Graham, J. B., and Graham, R. M., Surg. Gynecol. Obstet. **114**, 1 (1962).

21. Hughes, L. E., Kearney, R., and Tully, M., Cancer **26**, 269 (1970).

Received June 1, 1971. P.S.E.B.M., 1971, Vol. 138.