

Resistance to Common Viruses During Intralymphatic Injections of Tumor Cell Vaccines

Correlation with Circulating Cytokines

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Patients with advanced malignancies who received intralymphatic injections of irradiated tumor cell suspensions ("vaccines") were unexpectedly found to be resistant to common viral diseases; 17 patients with a documented past history of viral infections who have been observed for 48 to 148 months (median 108 months), were analyzed. The resistance to viruses was found to correlate closely with the presence, in the serum, of certain cytokines. Specifically, the interleukins, -2, -6, -8 and interferon-gamma, at low but sustained levels appeared to be possibly responsible for the nonspecific protection against viral infections obtained by intralymphatic injections of cellular material. These findings suggest that viral infections in normal or immunosuppressed individuals at particular risk might be prevented by treatments aimed at attaining very modest levels of certain cytokines.

Key Words: Intralymphatic immunotherapy—Viral resistance—Cytokines.

Since 1976 at UCLA, 280 patients with advanced malignancies have received four or more intralymphatic injections of various tumor cell suspensions as experimental treatment (1,2). The attention was focused on cancer response and side effects, particularly potential autoimmune diseases, anaphylactic reactions, and transmitted hepatitis. Adverse effects were monitored in 3,012 patient-months at risk, with a median follow-up of 10 months (3-126 months) and a total number of 2,153 injections: 94 patients survived at 12 months, 66 patients survived at 24 months, and 22 patients survived at 36 months. The singular lack of intercurrent viral infection in this immunocompromised population became gradually apparent, particularly during the seasonal outbreaks of common viral infections in their families, although, at the time, the incidence of viral infections was not specifically recorded.

Because of the impression that there may be a correlation between intralymphatic cellular injections and the resistance to viruses, a careful analysis of the incidence of viral infections before and after the beginning of intralymphatic injections of tumor cell suspensions was therefore initiated in 1988. A total of 17 patients were chosen for their past history of viral infections, with a follow-up of 48 months or more. The 17 patients were observed for a total of 1,783 patient-months for occurrence of viral infections (median: 108 months; range: 48-148 months). To our knowledge they represent a unique group of patients who have received multiple intralymphatic injections of cellular material. They are the subject of this report.

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PATIENTS AND METHODS

Selection of Patients for the Study

Criteria for patients' inclusion were (i) follow-up of 48 or more months, (ii) availability of serial frozen serum samples, and (iii) past history of common viral infections: "influenza" syndrome, common cold, recurring herpes simplex type I, averaging 1.9 (range: 1-3 viral infections) per year. This information was based on patient verbal reports, except for a patient with frequently recurring herpes who was followed at the dermatology clinic. Written consent was obtained from each patient. The subjects were studied with a protocol annually reviewed and approved by our institution's Human Subject Protection Committee.

Tumor Cell Vaccine Preparation

Patients received irradiated vaccines of either established cell lines M14 and 100P₃ from the Department of Surgical Oncology, UCLA, or BT-20, U2OS, and SkBr₃ from the American Tissue Culture Collection (Maryland), or autologous cells from the patients. Autologous cell lines were prepared from fresh sterile tumor specimens that were minced and passed through a mesh wire to a cell suspension. Cells were washed twice in Hank's Balanced Salt Solution (Irvine Scientific) and viability checked using the trypan blue (Irvine Scientific, Santa Ana, California) dye exclusion technique. Then 5×10^5 viable cells were seeded into a T-75 flask (Falcon) in triplicates. Fresh frozen cells and cell lines were stored frozen in liquid nitrogen at a concentration of 2×10^7 /ml. Immediately prior to use, the frozen cultured cells were thawed rapidly, washed, counted, and suspended in a final concentration of 5×10^6 viable cells/ml. In sterile lactated Ringer's solution (Baxter) and irradiated to 100 Gy in a Gammacell 220 (Atomic Energy Canada, Ottawa, Canada) for approximately 25 minutes. All preparations used as vaccines were tested for bacterial, fungal, and mycoplasma contaminant by conventional microbiologic techniques.

Immunotherapy

An average of 2×10^7 cells in 4 ml of saline per treatment were injected in the lymphatic afferents, which were surgically exposed and cannulated on the dorsum of the feet or the hands under local anesthesia (lidocaine 2%). When possible, cultured cell lines were used; otherwise, frozen "fresh" tumor was injected. Treatment was given monthly during the first year, or longer if there was persistent malignancy. In patients who were apparently free of cancer the interval between treatments was gradually increased to 6 months or the injections stopped.

Serum samples were obtained on the day of injections, every 1 to 6 months, then on follow-up report,

when immunotherapy was stopped. All samples were stored at -20°C until assayed. Then, 17 sera per assay were tested in duplicate and the mean value was calculated. A total of 131 sera were analyzed.

Cytokine Assays

Interleukin (IL) 1, alpha and beta, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8 levels in sera were measured using a commercial "Quantikine" (R&D Systems, Minneapolis, Minnesota) and interferon-gamma (IFN γ) by an IFN γ kit (Endogen, Inc., Boston, Massachusetts) according to the manufacturer's instructions. They employ the quantitative "sandwich" enzyme immunoassay with monoclonal antibody specific for a cytokine coated onto the microtiter plate wells. Standards and samples were pipetted onto the wells that were washed following incubation. Bound cytokines were detected using peroxidase-linked specific polyclonal antibody. Following incubation and washing to remove any unbound antibody-enzyme, substrate was added and the intensity of the color measured by a spectrophotometric plate reader. The concentration of the cytokine in the unknown samples determined by minimum detectable levels (in picograms per milliliter) were 0.2 pg/ml for IL-1 α , 0.3 pg/ml for IL-1 β , 10.0 pg/ml for IL-2, 8.9 pg/ml for IL-3, 4.1 pg/ml for IL-4, 0.35 pg/ml for IL-6, 6.0 pg/ml for IL-7, 18.1 pg/ml for IL-8, and 5 pg/ml for IFN γ .

Interferon- α (IFN α) levels in sera were measured by radio immunoassay using a commercial NK-2 IRMA kit (Celltech, Watertown, Massachusetts) in accordance with manufacturer's instructions. Standards and samples were pipetted into a 60-well tray. Sheep anti-interferon coated beads were added to the standard and samples. Beads were washed following 4-hours incubation. Bound IFN α was detected using ¹²⁵I monoclonal antibody tracer (NK-2). Following 18 hours incubation and washing to remove any unbound tracer, beads were counted in the gamma counter for 60 seconds. The concentration of IFN α in the unknown samples was determined by comparing the CPM counts of the samples to the standard curve. Minimum detectable level was 26 U/ml.

RESULTS

As part of their treatment, 17 patients with advanced malignancies underwent repeated intralymphatic injections of various irradiated tumor cells. Autologous or allogeneic lines of the same histologic type as the patient's tumor were used (Table 1). During the course of immunotherapy, none of the patients had relapses of previously occurring viral infections and in fact did not develop viral diseases of any kind. Serum samples taken prior to immunotherapy had no detectable cy-

TABLE 1. Patient characteristics

Patient	Sex	Age	Diagnosis	No. of ILCI	Cells used	F/U (months)	Cancer status	STR
1	M	29	Osteogenic sarcoma	61	LAO1/U205	66	DOD	+
2	F	28	Thyroid carcinoma	56	DRO81-1/HR081-1	109	DOD	+
3	F	19	Melanoma	77	M14	110	DOD	+
4	F	62	Breast carcinoma	45	BT20/SKBR 3	132	DOD	+
5	M	26	Melanoma	66	M14	136	AWD	+
6	M	69	Melanoma	101	M14	146	AWD	+
7	M	65	Melanoma	54	M14	134	A, Free	+
8	M	46	Melanoma	35	M14	144	A, Free	+
9	M	58	Melanoma	45	M14	146	A, Free	+
10	F	62	Squamous cell Ca	30	100P3	148	A, Free	+
11	M	36	Squamous cell Ca	6	100P3	112	A, Free	+
12	M	57	Squamous cell Ca	6	100P3	74	A, Free	+
13	M	53	Melanoma	7	M14	85	A, Free	+
14	F	79	Melanoma	25	M14	61	D, Free (autopsy)	+
15	M	52	Squamous cell Ca	7	100P3	73	A, Free	+
16	F	23	Melanoma	26	Autologous	61	A, Free	+
17	M	69	Thyroid carcinoma Anaplastic	24	Autologous	48	A, Free	+

Ca, cancer; DOD, dead of disease (cancer); AWD, alive with disease; A, Free, alive free of cancer; D, Free, dead free of cancer; STR, skin test reactivity; ILCI, Intralymphatic cellular injection.

tokines, with the exception of IL-8 in one patient. Following initiation of immunotherapy all patients developed detectable serum levels of IL-2, generally associated with IL-6, IL-8, or γ IFN (Table 2). In contrast, IL-1, IL-3, IL-4, IL-7, and TNF β were not detected, either before or after immunotherapy. IFN α assays were found to be unreliable. Cytokines were detected 15 to 39 days (median: 24 days) after the beginning of immunotherapy and persisted, as long as the intervals between injections were 3 months or less. In 3 patients the intervals between treatments were increased to 6

months and in 5 other patients the treatments were interrupted for 48 months or more. In 6 of these 8 patients, cytokine levels became undetectable and in all 6 patients, viral infections recurred. Of the other 2 patients free of viral diseases, one is receiving annual influenza vaccinations by his general practitioner and has no detectable cytokines, the other maintains detectable levels of γ -interferon and is receiving intralymphatic tumor cell vaccine every 6 months. The relationship between viral infections, cytokine levels, skin test reactivity, and tumor cell vaccine injections is shown in Fig. 1 for one of these patients. A viral relapse after 12 years was preceded by a drop in cytokines to undetectable levels. Anti-influenza A, B, and C antibodies were evaluated in 14 patients during immunotherapy. Two serum samples, approximately 1 year apart were assayed for each patient. No correlation between the lack of viral disease and antibody levels could be identified. However, the occasional elevation of antibodies suggested that patients had been exposed to viruses without clinical expression (Table 3).

Immunoglobulins G antibody titers against herpes simplex viruses (HSV1 and 2) were elevated in 16 patients and negative in one. Immunoglobulin M antibodies were negative in all patients during immunotherapy. This is consistent with exposure to viruses in most patients and disease in none.

Autoimmune tests and cold agglutinin titers remained negative in all patients.

DISCUSSION

Intralymphatic injections of irradiated tumor cells appeared to induce a state of resistance to common

TABLE 2. Cytokine detection before and during immunotherapy^a

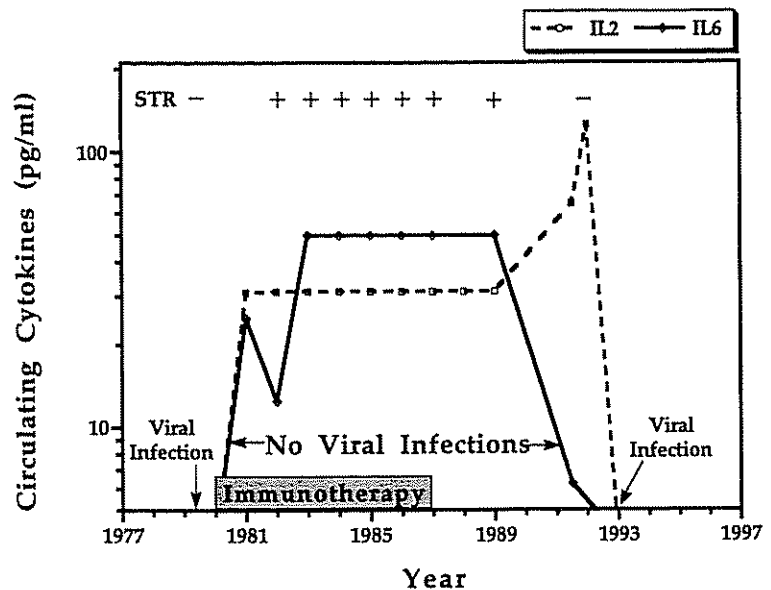
Patient no.	Before immunotherapy	During immunotherapy ^b			
		IL-2	IL-6	IL-8	INF γ
1	Undetectable	62.5	62.5	92.8	
2	NR	62.5		31.3	>10
3	NR	62.5	62.5	187.5	>10
4	NR	62.5		187.5	>10
5	Undetectable	31.5	25	187.5	>10
6	Undetectable	62.5	25	187.5	10.4
7	Undetectable	31.5		187.5	
8	Undetectable	62.5	12.5		>10
9	Undetectable	31.3	6.5		>10
10	Undetectable	62.5	50		
11	Undetectable	62.5	6.25	31.3	>10
12	Undetectable	31.3	6.25	92.8	
13	Undetectable	31.3	3.3	187.5	>10
14	Undetectable	62.5	12.5	750	
15	Undetectable	31.3			
16	Undetectable	31.3	12.5	31.3	400
17	IL8:92.8	31.3	6.25	31.3	

NR, sera not retrieved; IL, interleukin.

^a Interleukin-1, -3, -6, -7, interferon- α , and tumor necrosis factor-beta were undetectable in all samples.

^b Titers are in picograms per milliliter of serum.

FIG. 1. Correlation between serum cytokine titers and resistance to viruses (patient 10). STR, skin test reactivity.



viral infections, which was sustained in all patients, if the intervals between injections was less than 3 months. This effect did not appear to be related to specific antiviral antibody production but correlated closely with the presence of low sustained levels of endogenous cytokines, in particular IL-2, IL-6, IL-8, and $INF\gamma$.

The detection of these lymphokines and not of others (IL-1, IL-4, IL-7, $TNF\beta$) does not necessarily mean that they are the only cytokines produced. Detection of cytokines in serum is complicated by their varying half-lives, levels of soluble receptors, assays sensitivity, and the growing number of newly discovered cytokines. However, the serum cytokines profile, that is, IL-2 and INF as opposed to IL-4 production is generally consistent with enhanced cell-mediated immunity, which is in keeping with the fact that intralymphatic injections were previously found to enhance skin test reactivity (1).

TABLE 3. Antibody titers to influenza viruses, indicating exposure to viruses at undetermined time

Antibodies present against	No. of patients with titers > 1:8*	
	First sample	Second sample
Influenza virus		
A	4	5
B	1	0
C	2	3
Influenza viruses A and C	1	0
Influenza viruses A and B	2	0
Influenza viruses A, B, and C	3	2
Negative	1	4

* Samples were taken 1 year apart during cellular immunotherapy.

Nonspecific "viral interference" has been observed following viral immunization (3) and explained as due to interferon production (4,5). The therapeutic use of interferons has been impaired by the delay in their administration while viral infections are already overt, by the substantial side effects of the high doses necessary to be effective at this stage of the disease (6), and also, perhaps, because other cytokines may be required to achieve optimal effect (7). In the current study, low levels of circulating lymphokines, already present at the time of viral exposure, may have been sufficient to inhibit viral replication and prevent diseases. Relatively low levels of endogenous cytokines have been found to protect against vaccinia infections in children (8). It has been demonstrated that interferon-alpha functions effectively as a prophylactic agent against a variety of viruses in animals (9-11), even when immunosuppressed (12).

Others have observed that C8166 cellular injections in macaques induced unexpected protection against simian immunodeficiency virus (SIV) without production of specific antibodies to SIV, and a correlation was found with the titers of anti C8166 antibodies (13). This protection appears to have been caused by antibodies directed against normal cellular antigens present at the virion surface. Cytokine levels were not reported.

In the present analysis, anticellular antibodies were not evaluated. It cannot be ruled out that they could block viral receptors on the target cells (steric hindrance), but the same protection was obtained by various cells, including autologous, which would tend to weaken this hypothesis somewhat. Tumor-related antigens or contaminants are unlikely to be the trigger of viral resistance, given the often observed lack of cross-

reactivity of skin test to various cell lines (unpublished data). All cell lines were regularly screened for bacterial, fungal, and mycoplasma contaminations, which were never detected, and no viral inclusions were seen on routine electron-microscopic testing of the cellular vaccines.

Particularly fascinating was the persistence of the antiviral state and sustained cytokine levels for 3 months following "vaccination." In some patients this may persist longer, but in most, the refractory state fades without repeated injections. The intralymphatic route may be a critical element in achieving sustained serum cytokines level and viral resistance. Lasting retention of many soluble antigens, allogeneic or autologous cellular material in lymph nodes, has repeatedly been reported (14,15). It is conceivable that, in this site, cytokine production can be maintained for longer periods of time than by other routes.

It is suggested that nonspecific protection against a broad variety of viruses may be achieved by treatment that induces and maintains low levels of certain cytokines. ©

REFERENCES

1. Juillard GJF, Boyer P, Yamashiro CH. A phase I study of active specific intralymphatic immunotherapy (ASILI). *Cancer* 1978;41:2215-25.
2. Weisenburger T, Jones PC, Ahn SS, et al. Active specific intralymphatic immunotherapy in metastatic malignant melanoma: evidence of clinical response. *J Biol Response Modifiers* 1982;1:57-66.
3. Findley GM, Mac Callum FO. An interference phenomenon in relation to yellow fever and other viruses. *J Pathol Bacteriol* 1937;44:405-24.
4. Isaacs A, Lindenmann J. Virus interference. I. The Interferon. *Proc R Soc Lond* 1957;147:258-63.
5. Isaacs A, Burke D. Mode of action of interferon. *Nature* 1958;464:1073-6.
6. Itri LM. The interferons. *Cancer Suppl* 1992;70:940-4.
7. Merigan TC. Is recombinant interleukin 2 the best way to deliver interferon gamma in human disease? *J Interferon Res* 1987;7:635-9.
8. Petralli JK, Merigan TC, Wilbur JR. Action of endogenous interferon against vaccinia infection in children. *Lancet* 1965;2:401-4.
9. Denys P Jr. Protective effect of interferon in rats infected with sindbia virus. *Lancet* 1963;2:174.
10. De Somer P, Prinzie A, Denys P Jr, Schonne E. Mechanism of action of interferon. *Virology* 1962;16:63-70.
11. Lampson GP, Tyrell AA, Sretnen MM, Hillman MR. Purification and characterization of chick embryo interferon. *Proc Soc Exop Biol NY* 1963;112:468.
12. Neuman-Haeflin D, Shrestha B, Manthey KF. Effective antiviral prophylaxis and therapy by systemic application of human interferon in immunosuppressed monkeys. *J Infect Dis* 1976;133(Suppl):A211-6.
13. Stott, EJ. Anti-cell antibody in macaques. *Nature* 1991;353:393.
14. Juillard GJF, Boyer PJ, Niewisch H, et al. Distribution and consequences of cell suspensions following intralymphatic infusion. *Bull Cancer (Paris)* 1979;66:217-28.
15. Bubbers JE, Paman BA, Juillard GJF. Induction of canine in vitro reactivity to allsantigen following intralymphatic immunization. *Bull Cancer (Paris)* 1981;68:332-7.