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Immunology

Induction, establishment *in vitro*, and characterization of functional, antigen-specific, carrier-primed murine T-cell lymphomas

(radiation leukemia virus/*in vitro* infection/T helper cells)

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ABSTRACT **T cells were isolated from spleens of C57BL/Ka/Thy 1.1/Lb mice immunized with 2,4-dinitrophenylated keyhole limpet hemocyanin (DNP-KLH). The mice were infected *in vitro* with radiation leukemia virus and injected intrathymically into congenic C57BL/Ka (Thy 1.2) recipients. Within 3–4 months, seven thymic lymphomas developed, five of which were of donor type. The donor lymphomas were explanted and permanent cell lines were established *in vitro*. These lymphoma cells are capable of providing antigen-specific carrier (KLH)-primed help to DNP-primed B lymphocytes in secondary antibody production to DNP-KLH. They also enhance the secondary antibody response of whole primed spleen cell populations to DNP-KLH. The availability of these immortal clonable populations of immunofunctional neoplastic T lymphocytes should facilitate biological and biochemical investigations of lymphocyte interactions during synthesis of antibody to thymus-dependent antigens.**

The immune system is under regulation and control of many subpopulations of T lymphocytes whose functions have been poorly understood due to the unavailability of homogeneous monoclonal T cells *in vitro*. Establishment of antigen-specific T-cell lines is as important for studying T-cell functions as myelomas have been for studying B cells. However, whereas many monoclonal B-cell-derived myelomas are now available, no antigen-specific T-cell tumor has as yet been established. The somatic cell hybridization technique introduced by Köhler and Milstein (1) has been used successfully to produce many T-cell hybrids (2–4). In only two cases, however, have these hybrids yielded a functional product in the form of antigen-specific suppressor factors (5, 6). No other functions have been isolated and preserved.

We now describe an approach to establishing T-cell-derived tumors whose antigen specificities are determined prior to the initiation of malignant transformation. Lieberman and Kaplan (7) reported that lymphocytes infected *in vitro* with radiation leukemia virus (RadLV) (8, 9) can produce lymphomas after injection into histocompatible mice. Most of these lymphomas are of donor cell origin, can be grown *in vitro* as permanent cell lines, and bear cell surface antigens typical of T lymphocytes (27).

The method employed here for obtaining antigen-specific T-cell tumors involves: selection of T cells bearing Thy 1.1 antigen from spleens of immunized mice; *in vitro* infection of these cells with RadLV; injection of infected cells into congenic Thy 1.2 recipients; establishment of resultant donor-type lymphomas *in vitro*; and screening of the successfully established cell lines for antigenic specificity and function.

Using this method, we have obtained five T-cell lymphoma lines from spleens immunized to 2,4-dinitrophenylated keyhole limpet hemocyanin (DNP-KLH). They all appear capable of replacing carrier-primed T-helper cells in cooperative interactions with hapten-primed B cells, leading to secondary anti-DNP plaque-forming-cell (PFC) responses *in vivo*. When added to primed spleen cells in adoptive transfer experiments, they greatly enhance secondary anti-DNP-PFC responses. Both of these functions are antigen specific.

MATERIALS AND METHODS

Mice. C57BL/Ka (Thy 1.2) mice, 6–8 weeks of age, were x-irradiated with a dose of 450 roentgens (1 roentgen = 2.6×10^{-4} C/kg) and used as recipients of infected T lymphocytes. Congenic, C57BL/Ka/Thy 1.1/Lb mice ("BL/1.1" for convenience) immunized at 6 weeks of age were used as spleen cell donors at 5–6 weeks after immunization.

Virus. RadLV was prepared as a 20% cell-free extract of RadLV-induced C57BL/Ka thymic lymphomas in phosphate-buffered saline (P_i/NaCl), as described (9).

Isolation and Infection of Antigen-Primed T-Cells. BL/1.1 mice immunized 5–6 weeks earlier were given 5 mg of hydrocortisone sodium succinate (Solucortef, Upjohn) intraperitoneally, 48 hr prior to sacrifice and removal of the spleen. Cell suspensions were made in P_i/NaCl with 5% fetal calf serum (FCS). Erythrocytes were lysed in 0.83% NH_4Cl in distilled water for 3 min and the cells were washed three times in P_i/NaCl/5% FCS. Resuspended in RPMI 1640 medium with 10% FCS, the cells were loaded on tightly packed nylon wool columns (10), using approximately one spleen per column. After 1 hr of incubation at 37°C, the nonadherent lymphocytes were flushed out with 20 ml of warm medium. Recovery was between 26% and 30%, and by microcytotoxicity testing the recovered cell suspension contained more than 90% Thy 1.1-positive cells. These cells were then pelleted, incubated with RadLV at 37°C for 1 hr, washed repeatedly in large volumes of medium to eliminate unattached virus, and injected intrathymically into C57BL/Ka (Thy 1.2^+) recipients.

Establishment of T-Cell Lymphoma Lines *In Vitro*. When lymphomas became evident in mice, involved spleens, lymph nodes, and ascites fluid (when present) were removed under sterile conditions. Cell suspensions were made in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 20 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics, by mincing the tissues and passing the crude suspensions through nylon screens. After two washes, cell suspensions containing at least 5×10^6 cells per ml were seeded in 5 ml of medium in 60-mm plastic culture dishes (Lux Scientific Corporation), and incubated at 37°C in an atmosphere containing 5% CO_2 in

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Abbreviations: RadLV, radiation leukemia virus; DNP, 2,4-dinitrophenyl; TNP, 2,4,6-trinitrophenyl; KLH, keyhole limpet hemocyanin; CGG, chicken gamma globulin; PFC, plaque-forming cell(s); P_i/NaCl, phosphate-buffered saline; FCS, fetal calf serum.

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humidified air. Cultures were fed daily by the addition of 1 ml of medium. Periodically part of the spent medium was replaced without disturbing the cultures. It was usually possible to begin subculturing at high cell densities about 2 weeks after initial explantation *in vitro*. After approximately 1 month, the cells were considered fully established. They were then passaged every 2–3 days by transferring small aliquots of cells from dense cultures into new dishes containing fresh medium (11). Samples from established cell lines were frozen after every 4–5 passages in medium containing 20% FCS and 10% (vol/vol) dimethyl sulfoxide. They are easily reestablished in culture after thawing.

Serology. Anti-Thy 1.2 serum (AKR anti-C3H) was kindly supplied by Israel Zan-Bar (Division of Immunology, Department of Medicine, Stanford University). Anti-Thy 1.1 serum (C3H anti-AKR) was made available by Irving L. Weissman (Department of Pathology, Stanford University). Anti-Ly 1.2 (C3H/An anti-CE/J thymocytes) and anti-Ly-2.2 [(C3H/An × C57BL/6-Ly 2.1)F_1 anti-ERLD] sera were obtained from F. W. Shen (Memorial Sloan–Kettering Cancer Center, New York). Rabbit anti-mouse myeloma IgG, kindly provided by Leonard Herzenberg (Department of Genetics, Stanford University), was used as the PFC-developing antiserum. All antisera had a high degree of specificity for the corresponding antigens in serological tests.

Low tox-M complement and rabbit anti-mouse T-cell serum were purchased from Cedar Lane Laboratories, Ltd. (Accurate Chemical & Scientific Corporation, Hicksville, NY). Cell surface antigens were detected by either a two-step microcytotoxicity assay (12, 13) or quantitative absorption followed by direct cytotoxicity. Details of these techniques have been fully described (14, 15).

The final results of direct microcytoxicity tests are expressed as cytotoxic indices computed as follows:

$$\text{Cytotoxic index} = \frac{\text{Dead cells with Ab and C} - \text{dead cells in control}}{100 - \%\text{ dead cells in control}} \times 100,$$

in which Ab is antiserum and C is complement.

The final results of absorption tests are expressed as:

$$\%\text{ specific absorption} = \frac{\%\text{ absorption by test cells} - \%\text{ absorption by negative cells}}{\%\text{ absorption by positive cells}}.$$

Electron Microscopy. Lymphoma cells were fixed in 2.5% (wt/vol) glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. Thin sections were examined in a Hitachi electron microscope at 80 kV.

Antigens and Immunization. Keyhole limpet hemocyanin (KLH) (Pacific Biomarine Supply Company, Venice, CA) was dialyzed for 48 hr against 4 liters of P_i/NaCl in the cold. After a preliminary centrifugation at 7,800 × *g* to remove precipitate, the supernate was centrifuged at 201,000 × *g* for 90 min to pellet the protein. The pellet was resuspended in P_i/NaCl and filtered through a 0.45 μm filter.

DNP_{10}-KLH was prepared by allowing KLH to react with a 40-fold molar excess of dinitrofluorobenzene as described (16). DNP-chicken gamma globulin (CGG) was obtained from Leonard Herzenberg. Purified CGG (United States Biochemical Corp., Cleveland, OH) was coupled to dinitrofluorobenzene to yield DNP_8-CGG. *Bordetella pertussis* vaccine containing 2×10^{10} heat-killed organisms was purchased from the Division of Biologic Laboratories, Department of Public Health, Commonwealth of Massachusetts (Boston, MA).

Mice were primarily immunized with one intraperitoneal injection of 100 μg of alum-precipitated DNP-KLH or DNP-CGG, mixed with 2×10^9 *B. pertussis* organisms in a total volume of 0.2 ml. Alum precipitation was carried out as described (17). Five to 6 weeks later the spleens of the mice were removed and suspensions were made of: (*i*) mixed hapten-primed B cells and carrier-primed T cells; (*ii*) carrier-primed T cells purified by passage on nylon wool columns; (*iii*) hapten-primed B cells after complete elimination of T cells with rabbit anti-mouse T-cell serum.

In some experiments carrier-primed T cells were obtained from spleens of mice immunized 7 days earlier with an intraperitoneal injection of 100 μg of KLH as a precipitate formed with alum, plus 2×10^9 *B. pertussis*.

***In Vivo* Adoptive Anti-DNP PFC Response.** Various donor cell combinations, as specified in the tables, were injected intravenously into 3- to 4-month-old syngeneic recipients irradiated with 650 roentgens 24 hr prior to transfer. The recipients were challenged immediately with 10 μg of aqueous DNP-KLH or DNP-CGG intravenously. Seven days later their spleens were removed, those of three to five mice per group were pooled, and DNP-PFC numbers were estimated by the assay described by Cunningham and Szenberg (18). Spleen cell suspensions were mixed with trinitrophenyl (TNP)-coated sheep erythrocytes (19) and guinea pig complement with or without developing antiserum. Direct plaques developed with complement only were attributed to IgM antibody-producing cells. They were subtracted from the total number of PFC estimated after the addition of developing antiserum. The remaining PFC were considered IgG antibody producers. Their number was estimated per spleen as well as per 10^6 cells.

Sheep erythrocytes without TNP were used as additional controls.

RESULTS

T cells from spleens of BL/1.1 mice immunized with DNP-KLH were infected *in vitro* with RadLV and injected intrathymically (10^6 cells per mouse) into C57BL/Ka (Thy 1.2) recipients, irradiated with 450 roentgens 24 hr earlier. Seven of 8 mice developed lymphomas within 3–4 months. Five of these tumors, all bearing the Thy 1.1 donor marker, were established *in vitro* and designated as TL2-18, TL2-25, TL2-26, TL2-29, and TL2-30. In addition, cell lines TL2-9 and TL2-11, established from lymphomas originating from splenic T cells immunized with DNP-CGG, were used as specificity controls. In some cases, a RadLV-induced lymphoma cell line, BL/VL_3, and a radiation-induced lymphoma cell line, BL/RL_{12} (27), were used as additional nonprimed T-cell controls.

T-Lymphoma Cell Lines Can Function as Carrier-Primed T Cells. The ability of lymphoma cell lines TL2-18, TL2-25, TL2-26, and TL2-29 to cooperate with DNP-primed B cells in generating an *in vivo* adoptive anti-DNP response was tested. The results obtained with these "carrier-primed" cell lines were compared with those obtained when the same primed B cells were mixed either with *in vivo* primed T cells or with nonprimed BL/VL_3 and BL/RL_{12} lymphoma cells. The data of two independent experiments (Table 1) reveal that all of the carrier-primed cell lines were able to cooperate to various degrees with primed B cells in the *in vivo* adoptive anti-DNP response. This function was seen only when the secondary challenge was with the hapten conjugated to a homologous carrier (KLH). Challenging the secondary recipients with DNP-CGG (experiment 2, Table 1) yielded no response. The nonprimed BL/VL_3 and BL/RL_{12} lymphoma cell lines failed to show antigen-specific helper activity in the same experiment.

Table 1. Tests of function and antigenic specificity of T lymphoma cell lines' anti-DNP PFC response

Exp.	DNP-KLH 1° B cells*	KLH 1° T cells† or cell lines‡	Secondary antigen: DNP-KLH	Secondary antigen: DNP-CGG	Indirect DNP-PFC: Per spleen	Indirect DNP-PFC: Per 10^6 cells	% of standard§
1¶	5×10^6	2×10^6 T	+		19,060	1221	100
		TL2-18	+		660	118	9
		TL2-25	+		540	147	12
		TL2-29	+		5,480	571	46
2¶‖	5×10^6	2×10^6 T	+		6,000	176.4	100
				+	0	0	0
		TL2-18	+		180	21.4	12
				+	0	0	0
		TL2-25	+		300	21.4	12
				+	0	0	0
		TL2-26	+		1,800	128.5	72
				+	0	0	0
		TL2-29	+		11,200	329.4	186
				+	0	0	0
		BL/VL_3	+		80	2	0
				+	0	0	0
		BL/RL_{12}	+		0	0	0
				+	80	2	0

* T cells were depleted by treatment with anti-Thy-1.2 plus complement.
† Nylon wool column-purified T cells were used as a source of helper activity.
‡ Lymphoma cell lines derived from DNP-KLH-primed spleen T cells.
§ Calculated on the basis of PFC/10^6 cells.
¶ B cells alone, T cells alone, lymphoma cells alone, or antigen alone gave no plaques.
‖ Sheep erythrocytes without TNP were not lysed in any of the groups.

Enhancement of the Secondary *In Vivo* Anti-DNP Response. In Table 2, it may be seen that when 4×10^6 lymphoma cells of lines TL2-25, TL2-26, TL2-29, and TL2-30 were co-transferred with 8×10^6 DNP-KLH-primed spleen cells and the mice were challenged with a secondary dose of DNP-KLH, the anti-DNP response at day 7 was much greater than that observed in recipients of DNP-KLH-primed spleen cells alone. This increase was not found with the TL2-11 or TL2-9 control cell lines, the presence of which may even induce suppression. When KLH-primed normal T cells were used in the same manner (data not shown), an increase in antibody production of up to 7-fold was observed. Challenge with a heterologous antigen, DNP-CGG, failed to stimulate antibody production.

Presence of Ly Antigens on the Functional Cell Lines. It was of interest to ascertain whether the functional activity of

Table 2. Effect of T lymphoma cell lines on the *in vivo* adoptive anti-DNP PFC response

Exp.	DNP-KLH-primed spleen cells	Lymphoma cells	Cell line	Secondary antigen: DNP-KLH	Secondary antigen: DNP-CGG	Indirect DNP-PFC: Per spleen	Indirect DNP-PFC: Per 10^6 cells	% of standard*
1	8×10^6	5×10^6	—	+		6,880	491	100
			TL2-25†	+		48,260	1,371	279
			TL2-26†	+		35,620	1,349	274
			TL2-9‡	+		5,520	404	82
2	8×10^6	4×10^6	—	+		398,400	7,114	100
			—		+	30	0.5	0
			TL2-25†	+		1,905,200	28,017	393
			TL2-25†		+	340	4	0
			TL2-26†	+		1,184,000	11,653	163
			TL2-26†		+	6	0	0
			TL2-11‡	+		400,000	3,787	53
			TL2-11‡		+	10	0	0
			TL2-9‡	+		333,600	1,895	26
			TL2-9‡		+	10	0	0
3	8×10^6	4×10^6	—	+		1,136,000	14,521	100
			TL2-25†	+		630,000	17,500	120
			TL2-26†	+		1,050,000	65,625	451
			TL2-29†	+		3,326,000	73,911	508
			TL2-30†	+		2,804,000	70,100	482
			TL2-9‡	+		620,000	15,500	106

* Calculated on the basis of PFC/10^6 cells.
† Lymphoma cell lines derived from DNP-KLH-primed spleen T-cells.
‡ Lymphoma cell lines derived from DNP-CGG-primed spleen cells.

Table 3. Antigenic phenotype of T lymphoma cell lines

T-lymphoma cell line	Ly-1.2 Direct cytotoxicity*	Ly-1.2 Absorption†	Ly-2.2, Ly-3.2 Direct cytotoxicity*	Ly-2.2, Ly-3.2 Absorption†	Modulation *in vitro*
TL2-18	27	23 (100)	100	100 (100)	Ly-1.2↓ Ly-2.2$^+$
TL2-25	58	100 (ND)	80	100 (ND)	Ly-1.2$^+$ Ly-2.2$^+$
TL2-26	81	100 (100)	81	63 (100)	Ly-1.2$^+$ Ly-2.2↓
TL2-29	71	100 (90)	100	75 (0)	Ly-1.2$^+$ Ly-2.2↑
TL2-30	100	100 (ND)	0	0 (ND)	Ly-1.2$^+$ Ly-2.2$^-$

* Expressed as cytotoxic index.

† Expressed as percent specific absorption. % specific absorption on the original tumors at the time of *in vitro* explantation is shown in parentheses. ND, not determined.

these cell lines is correlated with the expression of the Ly-1 and Ly-2 differentiation antigens on their surfaces. These antigens have been shown to be expressed in various combinations on normal T-cell subsets with specialized functions (20–22). The results of both direct cytotoxicity and absorption tests performed on the fully established cell lines, as well as on the original tumors at the time of *in vitro* explantation, are shown in Table 3. There appears to be a lack of correlation between the Ly phenotype of the cell lines and their apparent helper functions, which in the normal situation would be attributed to T cells of Ly-1$^+$, Ly-2$^-$, Ly-3$^-$ phenotype. Expression of these antigens can undergo modulation *in vitro*. This was especially striking in the TL2-18 and TL2-29 cell lines. TL2-29 was established *in vitro* after the original tumor that was frozen was passed once more *in vivo*. Modulation may also have taken place in the secondary recipient.

Morphology. Electron microscopy revealed that most of the lymphoma cells have a "lymphoblastic" appearance: the nuclear to cytoplasmic ratio is lower than that of normal thymocytes; the chromatin and nucleolar features and the cytoplasmic organelles (ribosomes, Golgi apparatus, lysosomes, endoplasmic reticulum) are similar to those of normal lymphoblasts (23). One can also observe variable proportions of "immunoblast"-like lymphoid cells (24). They represent 1–2% of the total in TL2-9, TL2-25, TL2-26, and TL2-29 lines but are as high as 8% in TL2-18 and 19% in TL2-30. They are characterized by a very large "compact" nucleolus that is attached to the nuclear membrane. This immunoblastic appearance has not been seen in our virus-induced BL/VL cell lines or radiation-induced BL/RL cell lines (27). It appears somewhat related to the Ly phenotype because the cell lines with the highest percentage of immunoblasts are those preferentially expressing only one Ly phenotype.

DISCUSSION

Establishment of permanent, antigen-specific T-cell lines is a prerequisite for detailed study of T-cell functions. In this paper we have reported an approach to this problem. By using RadLV to infect a selected population of T lymphocytes from spleens of mice immunized with DNP-KLH, we have induced a series of antigen-specific T-cell-derived lymphomas and have established them as permanent cell lines *in vitro*.

The fact that these lymphoma cell lines retain T-helper activity demonstrates that neoplastic transformation does not abrogate previously acquired immunological functions. Conversely, these observations confirm other reports (11, 15) that mature, fully immunocompetent, lymphoid cells are still susceptible to virus-induced neoplastic transformation. It was recently reported (25) that fully transformed thymic lymphoma cells can be also stimulated by antigen and made to function as normal helper T cells. These observations indicate that the mechanisms involved in expression of an immune function are not necessarily affected by transformation.

Considering that only one T lymphocyte per 10^3 cells has the specificity for a given immunizing antigen (26), it appears that antigen-specific T cells had an advantage over other cells for RadLV-induced neoplastic transformation. The probability that this antigen-activated cell population would become the preferential target for virus infection and transformation may have been enhanced by prior injection of hydrocortisone, which would have been expected to eliminate most immature T cells as competitive targets.

It is to be hoped that the method described in this paper can be successfully extended to the transformation of other preselected subpopulations of T cells with defined immunospecific functions. Mass culture of such functional T cells will permit detailed biochemical analysis of these T-cell-mediated functions, including the molecular nature of the putative T-cell receptor for antigen and its relationship to various membrane structures and cell surface markers. Lymphoma cell lines capable of an immunological function may also be at the right stage of differentiation to be partners of choice for hybridization with normal T cells. A transformed cell that has retained a particular immune function may make the preservation of the parallel normal T-cell function in hybrids more likely than has so far been observed.

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