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Lymphocyte-mediated Cytotoxicity: Mechanisms and Relationship to Tumor Immunity*1

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Abstract: Destruction of tumor cells *in vitro* by immune lymphoid cells may be based on several mechanisms, *i.e.*, (a) direct killing by sensitized cytotoxic thymus-dependent lymphocytes (CTL); (b) antibody-dependent killing by normal lymphoid cells carrying receptors for the Fc portion of IgG molecules: and (c) cell-mediated lysis by macrophages "armed" or activated by soluble factors released by sensitized T cells following interaction with antigen. In the present report, studies will be described which were aimed at the analysis of the role of CTL *in vitro* and *in vivo*. Two different approaches were used.

First, attempts were made to increase cytotoxic activities of immune cell populations by incubating and separating CTL from other irrelevant lymphoid cells. A combination of preincubation and separation techniques led to a considerable increase in cytotoxic activities of lymphoid cell populations, allowing the detection of CTL in spleens of C57BL/6 mice bearing syngeneic EL4 lymphoma cells. Second, *in vitro* generation of CTL was studied using mixed lymphocyte-tumor cell cultures (MLTC). Under appropriate culture conditions, CTL were formed in MLTC containing normal C57BL/6 spleen cells and irradiated RBL-5 lymphoma cells or moloney sarcoma virus (MSV)-induced sarcoma cells. Moreover, it was found that generation of CTL *in vitro* was at least 10-fold higher when spleens from mice carrying or having rejected an MSV-induced tumor were used instead of normal spleens as the source of responding cells, suggesting the presence of increased numbers of CTL precursors in the former animals. Together, these results suggest that two approaches should be used in attempts to evaluate the immune status of tumor-bearing individuals. Lymphoid cell populations should be tested for CTL using various cell purification procedures to obtain cell subpopulations enriched in effector cells. CTL precursors should be sought using methods for generation of CTL in

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MLTC. The relevance of *in vitro* findings to resistance to tumor growth will be discussed.

In attempts to understand the mechanisms leading to resistance against tumor growth, many recent studies have focussed on the *in vitro* analysis of humoral and cell-mediated immune responses to tumor-associated antigens. Following the demonstration that lymphocytes of immune individuals may be cytotoxic for target cells carrying the sensitizing membrane-bound antigens, the *in vitro* assay of lymphocyte-mediated cytotoxicity (LMC) was widely used to measure cell-mediated immunity to grafts and tumors. Also, it was tempting to assume that cytotoxicity of sensitized lymphocytes constituted the main mechanism of tumor immunity. However, further studies have shown that a number of important problems remain to be solved. In particular, it has now become clear that *in vitro* destruction of tumor cells by immune lymphoid cells may be based on several mechanisms, *i.e.*, on (a) direct killing by sensitized cytotoxic thymus-dependent lymphocytes (CTL), (b) antibody-dependent killing by normal mononuclear non-T lymphocytes (K cells) which carry receptors for the Fc portion of the IgG molecule, and (c) cell-mediated lysis by macrophages "armed" or activated by soluble factors released by sensitized T cells following interaction with antigen (*1*).

Little information is at present available concerning the significance of these *in vitro* mechanisms in terms of tumor immunity *in vivo*. Furthermore, it is obvious that the *in vitro* demonstration of LMC using whole lymphoid cell populations from tumor-bearing individuals gives little information about the type of effector cell involved, and cell separation and identification procedures are necessary to identify the cytotoxic mechanism. In the few studies where such procedures were applied, it was found that the relative role of the different effector mechanisms depended on the assay system used, the time of immunization or state of tumor growth, the source of immune lymphocytes, and other factors.

In order to analyse more closely the role of CTL *in vitro* and *in vivo*, recent studies in our laboratory have concerned with the separation, the physico-chemical characterization and the quantitative *in vitro* assay of CTL formed in the primary and secondary *in vivo* and *in vitro* response to alloantigens (*2–4*) and tumor antigens (*5, 6*). The present report summarizes studies of (a) the separation and identification of CTL in the EL4 mouse lymphoma system, (b) the analysis of LMC in the moloney sarcoma virus (SMV) tumor system, and (c) the primary and secondary *in vitro* generation of tumor specific CTL in the SMV system. Furthermore, the problem of the relevance of the *in vitro* findings to resistance to tumor growth will be discussed.

Separation and Identification of CTL in the EL4 Mouse Lymphoma System

In syngeneic tumor systems it is often difficult to detect cytotoxic lymphocytes, presumably due to low effector cell frequencies and/or inhibitory factors. The possibility to separate CTL from irrelevant cells in order to increase cytotoxic activities

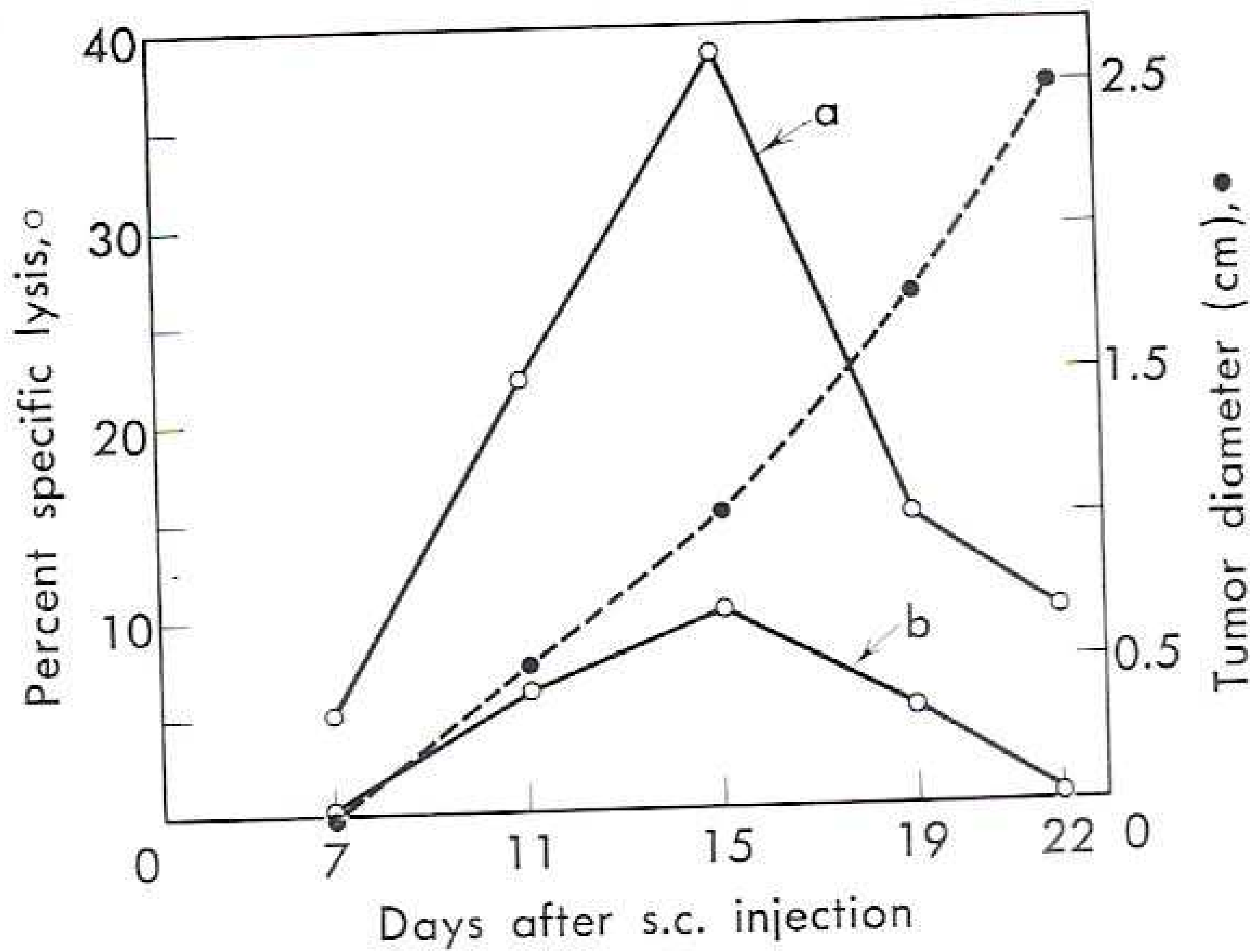

FIG. 1. Cytolytic activity of preincubated or BSA-gradient separated spleen cells of mice bearing a growing EL4 tumor. Spleen cells of C57BL/6 mice were collected at different time intervals after subcutaneous (s.c.) injection of 10^4 EL4 lymphoma cells and their cytolytic activity assayed, (a) after 24-hr preincubation, and (b) after 24-hr preincubation followed by BSA-gradient separation of low density (<1.08 g/cm^3) cells, against ^{51}Cr-labeled EL4 target cells at a spleen cell to target cell ratio of 100: 1 (24-hr incubation). No cytotoxic activity could be detected with spleen cells tested at time of collection (results not shown). ○ percent specific lysis; ● tumor diameter (cm).

of immune cell populations was suggested by studies in allograft systems. They had shown that early in the immune response, CTL were predominantly large cells (*7, 8*) of low density which could be separated from other cells by simple density gradient or velocity sedimentation procedures (*9, 10*).

In attempts to apply these findings to a weak syngeneic tumor system, spleen cells from C57BL/6 mice immunized by two intraperitoneal injections of irradiated (4,000 R) EL4 lymphoma cells were separated into a low and a high density fraction on a bovine serum albumin (BSA) density gradient. Cytotoxicity tests using a ^{51}Cr-release assay showed that the low density fraction containing 15–20% of the total cell number had a 5-fold increased cytotoxic activity as compared with the initial cell population (*5*). A comparable increase in activity was noted when immune peritoneal cells were tested following removal of phagocytic cells. Similarly, preincubation of immune spleen cells for 24 hr led to increased cytotoxic activities for reasons which are still not clear, and a combination of preincubation followed by density gradient separation led to a 30 to 100-fold increase. In an extension of these studies it was found that the application of these preincubation and separation techniques also allowed the detection of CTL in spleens of EL4 tumor bearing mice, *i.e.*, in a situation where it was not possible to detect cytotoxic lymphocytes in the direct ^{51}Cr-release assay (Fig. 1).

Treatment of immune spleen or peritoneal cells with anti-θ serum and C in order to selectively remove T cells completely abolished cytotoxicity, whereas passage through Ig-anti-Ig coated columns to remove surface membrane Ig carrying cells (bone marrow-derived cells (B cells)) and cells carrying Fc receptors (K cells) had no effect. These tests demonstrated the T-cell nature of the effector cells (*5*).

LMC in the MSV Tumor System

Mice injected with MSV form a sarcoma at the site of virus inoculation which may undergo complete regression within 2–3 weeks. Tumor regression is accompanied by the development of resistance to challenge with MSV. The tumor hosts form antibodies which react with membrane-bound antigens associated with MSV-induced tumors, and which are cytotoxic in the presence of complement or in the presence of normal lymphoid cells (K cells).

Studies of LMC in MSV immune mice showed a complex picture with the following characteristics. When assayed by the microcytotoxicity assay of Takasugi and Klein (*11*), cytotoxic activity of spleen cells was detectable within a few days after MSV injection, disappeared at the time of maximal tumor size, and then reappeared and persisted for several weeks after tumor regression (*12, 13*). The characterization of the effector cells indicated that both T-cell and non-T-cell cytotoxicity was involved before and shortly after tumor regression (*13, 14*). Late after regression, however, non-T cells only appeared to be responsible for the activity detected with this particular assay system.

When assayed by a ^{51}Cr-release test, cytotoxic activity was also detected within a few days after MSV injection, but it reached a peak at the time of maximal tumor size and then gradually declined (*15, 16*). Lymphoid cells collected late after complete regression of the tumor had no activity. Characterization of the effector cells showed that T cells only were responsible for the cytotoxic activity (*16, 17*) detectable with this assay system.

It is thus apparent that conflicting results may be obtained when the same lymphoid cell population is tested for LMC using two different assay systems.

In Vitro Generation of MSV Tumor-specific CTL

The formation of CTL with specificity for alloantigens can be readily induced in mixed lymphocyte cultures (MLC) *in vitro*. In recent studies in our laboratory

TABLE 1. Generation of CTL in Primary and Secondary MLTC as a Function of Time after MSV Inoculation

Origin of spleen cells in MLTC (days after MSV)	Lytic activity of MLTC cells: percent specific ^{51}Cr release from RBL-5 target cells in 3 hr at MLTC cell to target cell ratios of					
	100	30	10	1	0.1	Lytic units/culture
Normal	35	23	11	3	2	4
Immune (5 days)	85	57	33	5	2	39
Normal	66	44	15	2	3	8
Immune (14 days)	100	97	79	20	4	217
Normal	44	27	15	2	0	4
Immune (64 days)	82	75	72	17	1	155

Spleen cells from C57BL/6 mice collected at various times after MSV injection (immune cells) or spleen cells from uninjected C57BL/6 mice (normal cells) were mixed with X-irradiated RBL-5 lymphoma cells at a ratio of 25 : 1 (MLTC), incubated for 6 days and then assayed for lytic activity against ^{51}Cr-labeled RBL-5 target cells *in vitro*. Lytic units/culture were determined as described previously (*2*).

it was found that the MLC response of spleen cells from mice immunized 2–3 months previously with an allograft led to over 5-fold higher CTL activities than the MLC response of normal lymphocytes (*2*). This secondary type *in vitro* cell-mediated immune response was presumably based on the presence of increased numbers of CTL precursors (memory cells) in the spleens of the immune animals. Furthermore, a qualitative difference in the responsiveness of normal and immune spleen cells was suggested by the observation that immune cells responded almost as well to subcellular alloantigen preparations as to intact irradiated spleen cells, whereas normal spleen cells responded only to intact cells (*18*).

When similar experiments were carried out in the syngeneic MSV tumor system, it was found that CTL able to lyse ^{51}Cr-labeled Rauscher virus-induced RBL-5 leukemia cells were formed in cultures of C57BL/6 spleen cells and irradiated RBL-5 cells (mixed lymphocyte-tumor cell culture (MLTC)). Moreover, it could be dmeonstrated that the generation of CTL was at least 10-fold higher when spleens from mice carrying or having rejected an MSV-induced tumor were used as the source of responding cells (*6*), suggesting the presence of increased numbers of CTL in these animals. The results of an experiment in which the generation of CTL in primary and secondary MLTC was followed as a function of time after MSV inoculation are presented in Table 1.

It can be seen from these results that an increased, secondary type response could be observed with spleen cells collected as early as 5 days and as late as 64 days after MSV inoculation.

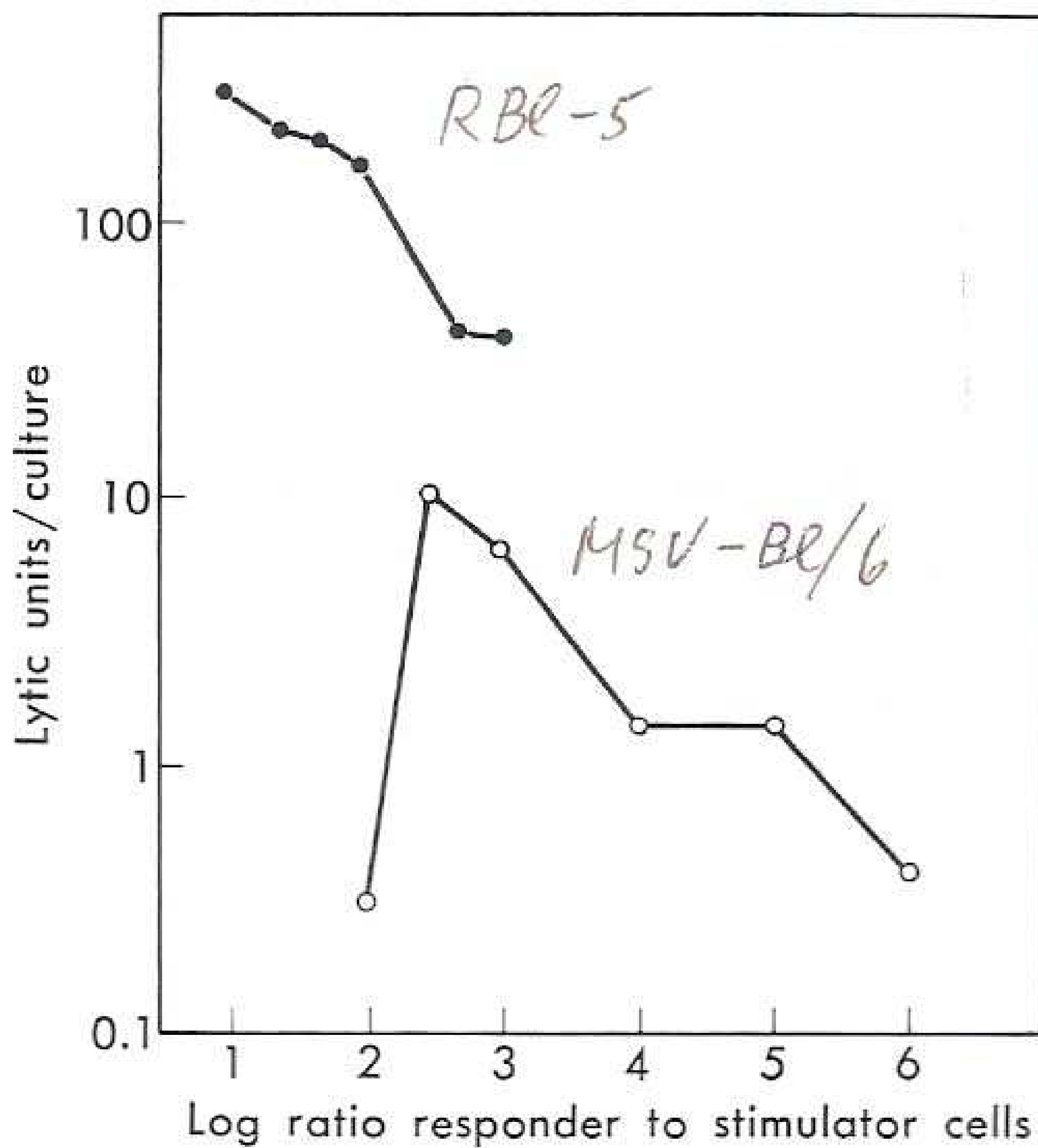

FIG. 2. Generation of CTL in secondary MLTC as a function of the ratio of stimulator to responder cells. MLTC were prepared by mixing spleen cells of C57BL/6 mice collected 26 days after rejection of an MSV-induced tumor (responding cells) with X-irradiated RBL-5 lymphoma or MSV-BL/6 sarcoma cells (stimulating cells) at various ratios. The MLTC were incubated for 6 days and the resulting cell population tested for cytolytic activity against ^{51}Cr-labeled RBL-6 target cells in a 3-hr assay. Lytic units/culture were determined as described elsewhere (*2*).

TABLE 2. Lytic Activity against ^{51}Cr-labeled MSV-BL/6 Target Cells of CTL Generated in Secondary MLTC Using Either the RBL-5 Lymphoma or the MSV-BL/6 Sarcoma as a Source of Stimulator Cells

Stimulating cells used in secondary MLTC	Lytic activity of secondary MLTC cells: percent specific ^{51}Cr release from MSV-BL/6 sarcoma target cells in 24 hr at MLTC cell to target cell ratios of			
	30	10	3	1
None	2	3	4	2
RBL-5 lymphoma	87	73	56	31
MSV-BL/6 sarcoma	71	36	20	1

Spleen cells from C57BL/6 mice collected following rejection of a tumor induced 26 days previously with MSV were mixed at ratios of 25 : 1 with X-irradiated (4,000 R) RBL-5 cells or at 300 : 1 with MSV-BL/6 cells in MLTC and incubated for 6 days. The cytolytic effect of the MLTC cells was then tested on ^{51}Cr-labeled MSV-BL/6 target cells at the ratios indicated.

The cytolytic activity of the *in vitro* generated CTL was demonstrable not only against RBL-5 target cells, but also against MSV-induced sarcoma cells, using both the ^{51}Cr-release test and the microcytotoxicity assay. Similarly, both RBL-5 lymphoma and MSV-BL/6 sarcoma cells were effective as stimulator cells in secondary MLTC, although at different optimal ratios (Fig. 2), inducing the formation of CTL which readily lysed ^{51}Cr-labeled MSV-BL/6 target cells (Table 2).

Studies of the specificity of target cell lysis by the primary and secondary MLTC cells showed that the reactivity appeared to be directed against surface antigens common to Rauscher virus- and Graffi virus-induced lymphoma cells and MSV-induced sarcoma cells (*6*).

As discussed before, mice which were resistant to MSV challenge late after rejection of an MSV tumor contained no detectable CTL in the spleen, while the present studies demonstrated secondary type MLTC responses suggesting the presence of increased numbers of CTL precursors as late as 64 days after MSV inoculation (Table 1). These findings draw attention to the fact that in attempts to evaluate the immune status of tumor-bearing individuals, immune lymphoid cell populations should not only be tested for CTL with the most sensitive methods available, perhaps including cell purification procedures as described above, but also for the presence of precursor or memory T-cells.

Relevance of In Vitro Findings to Resistance to Tumor Growth

Taken together, the studies so far described have shown that at least 3 LMC mechanisms observed *in vitro* may play a role in tumor immunity, namely killing by CTL, by antibody and K cells, and by immune non-T cells. Whether lysis by immune non-T-lymphoid cells is related to antibody-forming cells and K cells remains to be established. In addition, our studies now draw attention to the fact that memory T cells may be present in immune lymphoid cell populations and may be restimulated *in vitro* (or *in vivo*) to form CTL. (Complement dependent lysis by antibody is not being considered in this context).

To assign a definite *in vivo* function to a mechanism observed *in vitro*, one ap-

proach consists in studying the activity of a well-defined cell population following transfer into animals which are normally or artificially depleted of a given cell type. As shown above, separation procedures based on physico-chemical or immunological properties are available and may be useful to prepare cells enriched in a given cell category.

In the MSV tumor system, both serum (*19, 20*) and lymphoid cells from immune mice have been shown to confer protection *in vivo* (*19, 21–23*). Since the cytotoxicity assays of spleen cells from MSV immune mice described earlier had shown that late after tumor rejection the effector cells of LMC belonged to the non-T-lymphoid cells, it appeared possible that antibody and K cells were active in the cell transfer experiments. However, cell separation studies demonstrated that only T cells from immune spleens were able to protect irradiated recipients (*23*). As shown by our studies, immune spleen cells also contained increased numbers of CTL precursors, and it appears possible that these transferred memory cells had mediated protection, presumably by rapidly differentiating into CTL. This hypothesis is also supported by the finding that immune spleen cells restimulated *in vitro* in MLTC were able to confer resistance to mice injected with murine leukemia virus-induced leukemia cells (*24*). These MLTC populations contained a relatively large number of CTL, and it is tempting to speculate that protection was related to the direct action of these effector cells. However, immune T cells may also contain helper cells required for IgG antibody formation, and T cells which produce mediators acting on macrophages. Further studies are therefore required to more clearly define the relative importance of the various *in vitro* mechanisms of lymphocyte-mediated cytotoxicity for resistance to tumor growth.

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Discussion of Paper of Drs. Brunner et al.

DR. JERNE: Would you further define K cells?

DR. BRUNNER: K cells have not as yet been fully classified. They appear to be non-adherent mononuclear cells capable of lysing antibody coated target cells. They are most probably lymphoid cells distinct from T and B lymphocytes, although a few studies suggest they are B cells, *i.e.*, immunoglobulin carrying cells. In experiments involving antibody-dependent cell mediated killing of chicken erythrocytes, effector cells consist of monocytes and granulocytes as well as K cells, while human red blood cells are lysed by monocytes and granulocytes only. A common denominator of this type of effector cell is the presence of receptors for the Fc portion of the IgG molecule.

DR. HALPERN: In your separation and purification procedure to obtain a lymphocyte population enriched in cytotoxic cells, you discarded the high density fractions. Have you looked at the cytotoxicity of the cells which you removed?

DR. BRUNNER: We have thus far only examined the phenomenon of increased cytotoxicity following preincubation of the low density cell fraction alone or of the entire lymphocyte population which, of course, includes the high density fractions containing small lymphocytes. Preincubation of the light density fraction resulted in a sharp decrease in activity, whereas similar treatment of the entire population revealed a marked increase. We concluded that the high density fraction, which contains the cells we usually discard, includes precursor cells which may be activated by preincubation.

DR. HALPERN: We, as well as other investigators, have noted inhibitory interactions between lymphocytes and macrophages. Might this serve as an explanation for the fact that when one adds the cytotoxicity of the high density and low density cell population as measured separately in our laboratory, the sum is lower than the effect of each individual fraction?

DR. BRUNNER: By simple density gradient separation of spleen cells, we fortuitously were able to select out a population, comprising 15–20% of the total and located in the low density region, which contained a relatively high frequency of effector cells. This low density population also contained the macrophages, and it is therefore difficult to explain your observation by their inhibitory effect.

Dr. Hobbs: Experiments which O. Fakhri and I performed using the mouse plasmacytoma model may offer an explanation for the observation that the sum of individual immune activities was less than a given fraction. In this system we established heterologous rat attack on the mouse tumor, without the generation of T cell-mediated cellular immunity in the rat. Only 5 macrophages per tumor cell are required for effective killing, while 14 K cells, which are non-immune, non-adherent, lymphoid-like cells, are required for good cytotoxicity. However, a mixture of these two effector cell populations is not as good as either alone, perhaps because there is a limited amount of cell surface to which a tumor cell can attach.

Dr. Amos: In partial confirmation of some of your results, we found an extraordinarily high concentration of suppressor cells in the spleens of animals that had been intraperitoneally immunized with EL4 ascites tumor which is known to carry virus. We also observed insensitivity of lymphocytes to the target tumors produced by Rous sarcoma virus (RSV). Is the cell operative in your cultures a T cell or can it possibly be a B cell functioning *via* antibody production? Also, have you demonstrated the specificity of these effector cells?

Dr. Brunner: In the Moloney sarcoma virus system we have shown that the effector cells are T cells by using anti-θ serum plus complement as well as column separation techniques. Determination of specificity in the MSV system is complex in that the antigen is not clearly defined. We have demonstrated specificity by employing three types of MSV associated cells along with two unrelated control tumor cell lines, one syngeneic to C57BL/6, namely the EL-4 tumor used in the present system, and the P815 mastocytoma, which has DBA/2 histo-compatibility. Neither of the latter two served as target cells for the lymphocytes sensitized in primary or secondary mixed leukocyte tumor-cell cultures.

Dr. M. Hozumi (National Cancer Center Research Institute, Tokyo): Do you have any proposals concerning the details of the mechanism of target cell killing by lymphocytes? Have substances responsible for this cytotoxicity been isolated?

Dr. Brunner: Direct contact between the target and effector cells appears to be necessary, and no demonstration of any active principle released by lymphocytes in sufficient concentrations to be cytotoxic has yet been offered. If some factor is released by cytotoxic lymphocytes it must be so specific that it only kills the appropriate target cells and not itself or a syngeneic bystander cell. A cytotoxic T lymphocyte can apparently kill another cytotoxic T lymphocyte, but it cannot commit suicide.

Dr. F. Sendo (Cancer Institute, Hokkaido University School of Medicine, Sapporo): You showed that there is an increased number of precursors of cytotoxic cells in the tumor-bearing host. Why don't these cells differentiate into effectors? Also, did you check the activity of the culture medium in which spleen cells from the tumor-bearing animals were preincubated?

DR. BRUNNER: The reason why these precursor cells do not differentiate into cytotoxic lymphocytes may be because of suppressor mechanisms in the tumor-bearing mice or because these cells are saturated with antigen or complexes which inhibit this differentiation. Preliminary results with supernatants from our preincubation cultures have revealed an inhibitory effect in only some experiments.

DR. Y. KINOSHITA (Osaka City University School of Medicine, Osaka): Using lymph node cells obtained from Wistar rats injected with freeze-thawed Walker carcinosarcoma cells we were able to isolate two populations of lymphocytes by a combination of discontinuous density gradient centrifugation and a column packed with absorbent cotton. Our results published in the J. Natl. Cancer Inst., 1974, differ from yours in that the effector lymphocytes, assayed by microscopic observation and a ^{51}Cr release method, were here enriched in the highest density fraction. T cells from peripheral lymphoid tissues were more numerous in the high than low density populations. We proposed that, shortly after the last sensitization, some T cells transform into blastoid cells of low density and then further differentiate to form small lymphocytes, which have high cytotoxic activity and segregate in the higher densities. I, therefore, believe you have separated effector lymphocytes at the stage of blastoid cells or larger cells, while we performed this separation at the end of the maturation pathway.

DR. BRUNNER: In our experiments we took advantage of the fact that early in the immune response the cytotoxic lymphocyte is a low density large lymphoblast which will later transform into a small lymphocyte with higher density but is still cytotoxic. It is purely operationally that we segregated out the low density fraction to obtain a population with increased cytotoxic activity. I fully agree that cytotoxic lymphocytes exist in the high density fraction also, as well as precursor cells. Thus, depending on which type of cell you want, you can locate these effector cells in either fraction. It also depends on the system used for testing cytotoxic activity, as in the microplate assay, which usually requires a 48-hr incubation period, and even in course of the shorter lytic assays, differentiation may occur. Therefore, one may find most of the cytotoxic activity in high density cells either because they have had time to differentiate into cytotoxic lymphocytes, or because they already possess a high level of effector activity.