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Interpretation and In Vivo Relevance of Lymphocytotoxicity Assays

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The outcome of a short-term cell-mediated cytotoxic assay depends on the susceptibility of the target and the activation profile of the lymphocyte population. Certain cultured cell lines are highly sensitive to the lytic effect of lymphocytes of unimmunized donors, provided they have been derived from the same species; natural killing (NK) effect. This cytotoxicity seems to be independent of lymphocyte receptor-target antigen interaction but is likely due to some membrane property of the target (Ährlund et al. 1980). Specificity occurs only on the species level.

In the majority of studies which deal with characterization of the NK phenomenon one or a few lymphoblastoid cell lines are used as targets. With regard to NK sensitivity, cell lines fall into three categories, viz., those which are:

1. Sensitive in short term assays, with effector target ratios at and below 50:1. Extensive target cell damage is inflicted at low ratios which shows that the proportion of lymphocytes that can kill these targets is relatively high.
2. Sensitive only in long term assays.
3. Insensitive.

The difference between the first and second categories is probably quantitative. It seems that the lymphocyte population is heterogeneous with regard to the strength of the lytic function. Targets are characterized by a difference of the sensitivity to a lytic threshold which must be mounted by the lymphocyte. The difference between various targets is reflected in dose response experiments by the difference of the number of effector cells required to kill a certain number of targets. Increased effectivity can be achieved by manipulating the assay conditions. If a more prolonged target cell/lymphocyte interaction is permitted to take place, the cytotoxic potential of the effector population is elevated. Interferon (IFN) production appears to play a key role in this event (Santoli and Koprowski 1979).

A. Natural Killing

"Natural killing" is an operational designation. A certain regularity appears in the human system. In short term tests T cell derived lines are more sensitive than those derived from B cells (Ono et al. 1977). Studies with the B lymphoblastoid lines showed that tumor-derived lines are more sensitive than those derived from normal cells (Jondal et al. 1978). In this connection it may be noted that the presence of EBV genome in the cell does not seem to influence NK sensitivity. EBV-negative B tumor lines and their EBV-converted sublines (derived by superinfection of the EBV-negative line with the virus) displayed no difference in sensitivity (to be published).

Experiments in mice with alternating retransplantation and explantation of tumor cells showed a change in NK sensitivity depending on whether the same target cells were harvested from the mouse or from the culture (Becker et al. 1978).

The relatively higher sensitivity of cultured cells compared to directly explanted cells (Becker et al. 1978; De Vries et al. 1975) may indicate that NK-sensitive cells are eliminated in vivo. It is conceivable that NK sensitive variants arise de novo when the population is released from the selective pressure of killer lymphocytes. Alternatively, after explantation a change of cell membrane characteristics may occur.

The majority of human blood lymphocytes with natural cytotoxic potential were found to belong to the T cell series (Kaplan and Calleweart 1978). They are heterogeneous with regard to their surface marker characteristics, however (Bakács et al. 1978). The ranking order of different subfractions derived from the nylon nonadherent lymphocyte population with regard to their "specific activity", i.e., their cytotoxic potential on a per cell basis, is as follows:

1. Non-SRBC-rosetting (which contains cells reactive with anti-T serum) subset with and without demonstrable Fc receptors;
2. SRBC-rosetting cells with relatively low avidity E receptors and concomitant expression of Fc receptors;
3. Cells with low avidity E receptors without Fc receptors;
4. Cells with high avidity E receptors and Fc receptors; and
5. Cells with high avidity E receptors without Fc receptors (Masucci et al. 1980a).

The good NK activity of nude mice without thymuses has been taken as evidence for the non-T nature of the killer cells. However, immature or precursor T cells are present in these mice and induction of differentiation by thymus implantation resulted in decrease in NK activity (Herberman and Holden 1978).

Short term exposure of lymphocytes to IFN results in a considerably enhanced NK activity (Trinchieri et al. 1978).

The non-B cells of nude mice resemble the "null" and low avidity E rosetting cell category of man in that they have a high NK activity that can be enhanced by interferon treatment. These cells may have a role in vivo, since nude mice can reject grafts of virus infected tumor cells (Minato et al. 1979).

Assays for NK and interferon activated killing (IAK) differ only operationally, in that IAK involves IFN pretreatment of the effector cells. The two systems overlap. The results with lymphocytes of individuals with high NK activity are similar to IFN-activated lymphocytes from donors which function at a lower level of natural activity. Thus different persons can be assumed to be "preactivated" at different levels. The similarities between the two systems suggest that the rules emerging from IAK experiments are likely to be valid for the NK system as well. In view of the known prompt triggering effect of interferon for cytotoxicity, it is likely that this mechanism is important in the host response against virus infected and tumor cells.

Activation by interferon occurs before the clone of lymphocytes with antigen-specific receptors have the opportunity to enlarge. This cytotoxicity may therefore be the first active measure of the host defense by which altered cells are eliminated and virus spread is inhibited.

Interferon production is induced by viruses and by virus-carrying cells. In addition, interferon is produced during immune interactions between lymphocytes carrying specific receptors and the antigens. Thus, activation of nonselective cytotoxicity by virus-infected cells may occur through two mechanisms. The first is the effect of the virus and the second is the consequence of recognition of the altered cells and the virus by specific immunocompetent cells. The selective antigen-specific cytotoxicity appears lather, after proliferation of the clone with the specific receptor.

B. Generation of Killer Cells in Culture

The NK activity of lymphocytes gradually disappears under culture conditions (Masucci et al. 1980a). However, when lymphocytes are activated in culture by specific stimuli (e.g., in MLC), cytotoxicity is generated which affects not only targets related to the stimulus but also other cells that have no known antigenic relationship to it. In addition to alloantigens (Calleweart et al. 1978), calf serum (Zielske and Golub 1976), and some modification that occurs on the surface of cultured tumor cells (Martin-Chandon et al. 1975), EBV-transformed autologous and allogeneic lymphoid lines (Svedmyr et al. 1974) and PHA (Stejskal et al. 1973) can also trigger this type of cytotoxicity. In the allogeneic MLC system the activity was designated as "anomalous killing" (Seeley and Golub 1978). The activated lymphocyte can also affect certain targets that are NK resistant in short-term tests (Masucci et al. 1980a,b).

Since there is a quantitative correlation between generation of blastogenesis and the efficiency of the killing potential, (against K562, Molt-4, etc.) the latter can also be used as a measure of activation (Masucci et al. 1980a: Vánky et al. 1981).

The natural and the cultured activated killer (AK) cell populations show slightly different characteristics (Poros and Klein 1978). A relatively higher proportion of the AK cells adhere to nylon wool. The proportion of Fc receptor carrying killer cells in lower, and the FcR positive cells possess fewer or less avid receptors in the AK than in the NK system. T cells with high affinity E receptors are the least active both in the fresh (NK) and cultured (AK) populations.

Neither NK nor AK show the histocompatibility restriction phenomenon. The AK of a lymphocyte culture is not brought about by surviving NK cells but are triggered de novo. T cell populations depleted of the NK active subsets became cytotoxic on exposure to appropriate activating stimuli (Masucci et al. 1980); lymphocytes which have been kept in autologous plasma in vitro for several days and have lost NK activity can become cytotoxic when cultured further with K562 cells or exposed for short time to interferon (Poros and Klein 1978; Vánky and Argov 1980).

On the population level the following cytotoxicity systems can be generated in antigen-containing lymphocyte cultures:

1. Enlargement of the specific clone will result in cytotoxicity against
   (a) cells which carry the stimulating antigen and
   (b) cells which carry cross reactive antigens,
2. Transactivation will recruit lymphocytes with other specificities, and thus targets unrelated to the stimulus may be killed if the proportion of lymphocytes with receptors against their antigens is higher (Augustin et al. 1979).
3. Activated lymphocytes kill certain type of targets (cultured lines). This interaction is probably independent of antigen recognition.

Analysis of antigen-induced cytotoxic systems suggests that in any experiments the question of "specificity" on the effector level can only be asked if target cells of similar characteristics are used. As we have been proposed earlier (Martin-Chandon et al. 1975), the detection of specifically reactive cells is perhaps more meaningful at the level of the recognition step.

Awareness of these phenomena is important for interpretation of cytotoxicity experiments and to decide whether NK or AK is relevant in surveillance against virus-infected or transformed cells. The questions to be raised are as follows: Are lymphocytes which recognize the altered cell surfaces present in the lymphocyte population? Can these be activated by nonspecific means for cytotoxicity and can such cytotoxicity give an adequate protection? Is the viral infection or malignant transformation accompanied by changes of the plasma membrane which can interact with T lymphocytes similar to what is seen with cultured lines?

C. Killer Cells in the Blood of Acute Infectious Mononucleosis Patients

In view of the prompt triggering effect of IFN for the cytotoxic potential of lymphocytes and the wider cell panel affected by activated lymphocytes it is likely that the lytic effect of blood lymphocytes in these diseases is a consequence of activation. Infection with EBV imposes proliferation on B cells in vitro. It would be expected, therefore, that the lymphocytosis and the blasts in the blood of EBV mononucleosis are due to B cell proliferation. This is however not the case: the cells belong to the T lineage and B cells are few (Enberg et al. 1974). In fact, special measures (separation of subsets) had to be used to detect the EBV-infected B cells (Klein et al. 1975). During the acute phase when blasts are present in the blood, short term cytotoxicity of the lymphocytes is not restricted to NK sensitive targets (Svedmyr and Jondal 1975). These cells kill EBV-transformed lymphoblastoid B lines and also the ones derived from autologous lymphocytes. It is likely that in this cytotoxicity the recognition of an EBV-determined surface antigen on the target cells does not play a role but is due to the activation state of the T cells. The presence of T cell clones which recognize the EBV antigens, however, can be demonstrated later, after the acute phase subsided (Moss et al. 1978). The detection of such T cells during the acute phase would require experimental conditions in which the nonspecific effect of activated lymphocytes is eliminated. As we have shown before, the elimination of Fc receptor positive cells is not sufficient to achieve this with activated populations (Masucci et al. 1980a,b).

The autoreactive lymphocytes in the acute phase may be important for the recovery of the patient by limiting the proliferative tendency

of the EBV infected B cells. However, while in the majority of IM cases the activated T cells probably have a beneficial surveillance function, in some cases they may result in autoimmune phenomena of varying severity (Purtilo et al. 1979).

D. Autotumor Reactive Cells in Patients

In the course of experiments aimed to detect tumor-specific autoreactivities of patients with solid tumors we have performed short term cytotoxicity tests with blood lymphocytes against the autologous tumor biopsy cells [autologous lymphocytotoxicity (ALC)] (Vose et al. 1977). The outcome of these experiments indicated recognition of tumor cells in 28% of the cases. In an attempt to enhance the efficiency of the cytotoxicity test the lymphocytes were pretreated with IFN prior to the assay. This measure did not alter the results in the ALC but induced cytotoxicity against allogeneic tumor biopsy cells in 50% of the cases (Vánky et al. 1980b). Without IFN treatment, 5% of the allogeneic tests with lymphocytes of tumor patients and 14% with the lymphocytes of healthy donors were positive. The results were interpreted as an IFN-induced polyclonal activation of the lymphocyte population and manifestation of cytotoxicity by those T-cells which carry receptors against the histocompatibility antigens present on the particular target.

In order to impose more efficient stimuli for activation we have attempted to generate cytotoxic cells by cultivating blood lymphocytes with autologous tumor biopsy cells or use conditions that bring about lymphocyte activation such as MLC with the patient's lymphocytes as responders (Vánky et al. 1981).

In 19 MLCs in which the patients' lymphocytes were used as responders, seven generated autologous tumor cell killers and 11/13 damaged allogeneic tumor cells (unrelated to the reactants). The effects against the allogeneic tumor cells were either due to antigenic cross reactivities between the stimulator lymphocytes and the target tumor cells and/or transactivation of alloreactive lymphocytes with other specificities.

Assuming that there was no antigenic relationship between the stimulator allolymphocytes and the autologous tumor cells, the results can be interpreted in such a way that in 40% of cases autologous tumor recognizing lymphocytes were "transstimulated" in the MLC. K562 cells were regularly killed by all MLC effectors which provided the proof that activation took place in all cultures.

Autoreactivity by specific means, i.e., in the autologous mixed cultures containing lymphocytes and tumor cells, was generated in a higher proportion of tests (12 of 19).

The most effective system to generate killer cells against autologous tumor cells was thus the autologous mixed culture. Even in those cases in which autotumor killing was not generated the lymphocytes were activated by the encounter with tumor cells because they killed the K562 cells. This indicated that recognition of the tumor cells took place in the autologous mixed cultures.

Our results with the solid tumor cells are similar to those reported by Zarling et al. (1976) and by Sharma and Odom (1979) who used freshly harvested leukemia cells. Zarling and Bach (1979) mentioned that short term IFN treatment of the lymphocytes did not induce cytotoxicity against the autologous leukemia cells. This was achieved, however, when a strong stimulus was provided by confrontation with a pool of several allogeneic lymphocytes (Zarling et al 1976) or with soluble bacterial extract (Sharma and Odom 1979). It was also shown that autologous EBV-transformed lymphoblastoid cell lines could be killed by MLC-activated lymphocytes (Seeley and Golub 1978). Killers affecting autologous leukemia cells could be induced to proliferate by exposure to TCGF (Zarling and Bach 1979).

If cytotoxic cells will be available for therapeutical administration, similar considerations which concern drug or radiotherapy may have to be raised, i.e., in addition to the antitumor effects the specifically activated lymphocyte population may damage sensitive nonmalignant cells also.

The demonstration of the occurrence of lymphocytes which recognize the autologous tumor biopsy cells indicate that immunologic recognition of the autologous tumors is a reality and further research on this line is meaningful and important. On the basis of the in vitro experiments a more convincing therapeutic effect of nonspecific immunostimulation would have been expected than has been achieved. Modification of the therapeutic stra-

tegy is perhaps still a possibility which should receive further attention.

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References

Ährlund-Richter L, Masucci G, Klein E (1980) Somatic hybrids between a high NK-sensitive lymphoid (YACIR) and several low sesitive sarcoma or L-cell derived mouse lines exhibit low sensitivity. Somatic Cell Genet 6:89–99 – Augustin AA, Julius MH, Casenza H (1979) Antigen-specific stimulation and transstimulation of T-cells in long term culture. Eur J Immunol 9:665–670 – Bakács T, Klein E, Yefenof E, Gergely P, Steinitz M (1978) Human blood lymphocyte fractionation with special attention to their cytotoxic potential. Z Immunitaetsforsch Immunobiol 154:121–134 – Becker S, Kiessling R, Lee N, Klein G (1978) Modulation of sensitivity to natural killer cell lysis after in vitro explantation of a mouse lymphoma. J Natl Cancer Inst 61:1495–1498 – Calleweart DM, Lighbody JJ, Kaplan J, Joroszowski J, Peterson WD, Rosemberg JC (1978) Cytotoxicity of human peripheral lymphocytes in cell mediated lympholysis, antibody dependent cell-mediated lympholysis and natural cytotoxicity assay after mixed lymphocyte cultures. J Immunol 121:81–85 – De Vries JE, Meyerung M, Van Dongren A, Rümke P (1975) The influence of different isolation procedures and the use of target cells from melanoma cell lines and short term cultures on the nonspecific cytotoxic effect of lymphocytes from healthy donors. Int J Cancer 15:391–400 – Enberg RN, Eberle BJ, Williams RC (1974) T and B cells in peripheral blood during infectious mononucleosis. J Infect Dis 130:104–111 – Herberman RB, Holden HT (1978) Natural cell-mediated immunity. Adv Cancer Res 27:305–370 – Jondal M, Spina C, Targan S (1978) Human spontaneous killer cells selective for tumour-derived target cells. Nature 72:62–64 – Kaplan J, Calleweart D (1978) Natural killer cells express human T lymphocyte antigens. J Natl Cancer Inst 60:961–964 – Klein G, Svedmyr E, Jondal M, Persson PO (1975) EBV determined nuclear antigen (EBNA)-positive cells in the peripheral blood of infectious mononucleosis patients. Int J Cancer 17:21–26 – Martin-Chandon MR, Vánky F, Carnaud C, Klein E (1975) "In vitro education" on autologous human sarcoma generates nonspecific killer cells. Int J Cancer 15:342–350 – Masucci G, Poros A, Seeley JK, Klein E (1980) In vitro generation of K562 killers in human T-lymphocyte subsets. Cell Immunol 52:247–254 – Masucci MG, Klein E, Argov S (1980a) Disappearance of the NK effect after explantation of lymphocytes and generation of similar monospecific cytotoxicity correlated to the level of blastogenesis in activated cultures. J Immunol 124:2458–2463 – Masucci MG, Masucci G, Klein E, Berthold W (1980b) Target selectivity of interferon induced human killer lymphocytes related to their Fc receptor expression. Proc Natl Acad Sci USA 77:3620–3624 – Minato N, Bloom BR, Jones C, Holland J, Reid LM (1979) Mechanism of rejection of virus persistently infected tumor cells by athymic nude mice. J Exp Med 149:1117–1133 – Moss DJ, Rickinson AB, Pope JH (1978) Long term T cell mediated immunity to Epstein Barr virus in man. I. Complete regression of virus-induced transformation in cultures of seropositive donors' lymphocytes. Int J Cancer 22:662–668 – Ono A Amos DB, Koren HS (1977) Selective cellular natural killing against human leukemic T cells and thymus. Nature 266:546–647 – Poros A, Klein E (1978) Cultivation with K562 cells leads to blastogenesis and increased cytotoxicity with changed properties of the active cells when compared to fresh lymphocytes. Cell Immunol 41:240–255 – Purtilo DT, Paquin L, DeFlorio D, Virzi F, Sakhuja R (1979) Immunodiagnosis and immunopathogenesis of the X-linked recessive lymphproliferative syndrome. Semin Hematol 16:309–343 – Santoli D, Koprowski H (1979) Mechanism of activation of human natural killer cells against tumor and virus-infected cells. Immunol Rev 44:125–162 – Sharma B, Odom LF (1979) Generation of killer lymphocytes in vitro against human autologous leukemia cells with leukemia blasts and BCG extract. Cancer Immunol Immunotherp 7:93–98 – Seeley JK, Golub SH (1978) Studies on cytotoxicity generated in human mixed lymphocyte cultures. I. Time course and target spectrum of several distinct concomitant cytotoxic activities. J Immunol 120:1415–1422 – Stejskal V, Lindberg S, Holm G, Perlman P (1973) Differential cytotoxicity of activated lymphocytes on allogeneic and xenogeneic target cells. II. Activation by phytohemagglutinin. Cell Immunol 8:82–92 – Svedmyr EA, Deinhardt F, Klein G (1974) Sensitivity of different target cells to the killing action of peripheral lymphocytes stimulated by autologous lymphoblastoid cell lines. Int J Cancer 13:891-903 – Svedmyr E, Jondal M (1975) Cytotoxic effector cells specific for B cell lines transformed by Epstein-Barr virus are present in patients with infectious mononucleosis. Proc Nat Acad Sci USA 72:1622–1626 – Trinchieri G, Santoli D, Koprowski H (1978) Spontaneous cell-mediated cytotoxicity in humans: Role of interferon

and immunoglobulins. J Immunol 120:1849–1855 – Vánky F, Argov S (1980a) Human tumor lymphocyte interaction in vitro. VII. Blastogenesis and generation of cytotoxicity against autologous tumor biopsy cells are inhibited by interferon. Int J Cancer 26:405–411 – Vánky F, Argov S, Einhorn S, Klein E (1980b) Role of alloantigens in natural killing. Allogeneic but not autologous tumor biopsy cells are sensitive for interferon induced cytotoxicity of human blood lymphocytes. J Exp Med 151:1151–1165 – Vánky F, Argov S, Klein E (1981) Tumor biopsy cells participating in systems in which cytotoxicity of lymphocytes is generated. Autologous and allogeneic studies. Int J Cancer 27 – Vose BM, Vánky F, Klein E (1977) Lymphocyte cytotoxicity against autologous tumor biopsy cells in humans. Int J Cancer 20:512–519 – Zarling JM, Bach FH (1979) Continuous culture of T cells cytotoxic for autologous human leukemia cells. Nature 280:685–687 – Zarling JM, Raich PC, McKeough M, Bach FH (1976) Generation of cytotoxic lymphocytes in vitro against autologous human leukemia cells. Nature 262:691–693 – Zielske JV, Golub SH (1976) Fetal calf serum induced blastogenic and cytotoxic response of human lymphocytes. Cancer Res 36:3842–3846