Central Inhibition of Cellular Immunity to Leukemia L1210 by Isoantibody

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SUMMARY

Hyperimmune antibody (Ab) to leukemia L1210 inhibited the development of spleen cell-mediated immunity, as measured by an in vitro assay. Administration of 0.4 ml of Ab i.p. before i.p. challenge with tumor completely suppressed cellular immunity, but the same dose of Ab given after the tumor was relatively ineffective. The degree of suppression was not explainable entirely by killing of tumor cells. Synthesis of isohemagglutinins and cytotoxic antibodies was generally unaffected, further suggesting a “central” effect upon host lymphoid cells. Pretreatment of donor spleen cells with Ab in vitro or in vivo before i.v. injection into lethally irradiated recipients prevented the development of cellular immunity to a subsequent challenge with L1210. Addition of normal bone marrow cells partially overcame the immunosuppression in these recipients of Ab-treated spleen cells. The inhibitory activity of Ab resided almost entirely in the IgG fraction. It is suggested that this phenomenon represents “enhancement,” as defined in its broadest sense, of antibody-mediated inhibition of cellular immunity, with concomitant tumoricidal effects accounting for the failure of the leukemia to flourish.

INTRODUCTION

The interaction of humoral antibody and cellular immunity (delayed hypersensitivity) in the rejection of foreign tissues has been investigated primarily in systems where immunological enhancement can readily be demonstrated. Thus, skin grafts and solid tumors, particularly sarcomas, have been the main objects of study, where the effectiveness of treatment with antibody has been shown by extended graft survival or progressive growth of the tumor in immunologically incompatible hosts. Ascites leukemias in mice are generally resistant to true tumor enhancement, i.e., enhanced growth, presumably because of their high sensitivity to cytotoxic antibody (2, 19).

A number of immunological assays have been developed within the past decade that enable specific quantitative determination of spleen cell-mediated immunity as well as several types of serum antibodies. It is now possible to measure the cellular and humoral responses of the host under various conditions of antitumor therapy more precisely than survival data or measurement of the size of the tumor has previously permitted.

The studies reported here summarize an investigation of the effects of “therapeutic” courses of hyperimmune isoantibody upon the growth of leukemia L1210 in resistant allogeneic hosts. Although antibody does cause tumoricidal effects beneficial to the host in vivo, it may also lead to a selective specific abolition of cellular immunity that is operative, at least in part, at a central level, analogous to enhancement in other systems.

MATERIALS AND METHODS

Animals

DBA/2J (DBA) male mice, genetically H-2^d, were used for weekly passage of leukemia L1210. C57BL/6J (C57) male mice, H-2^b, were the recipients of L1210 in all experiments reported here, except those involving irradiated hosts. In the latter experiments, C57BL/6J X A/Jax F, (BAF1) male mice were used, because of a high incidence of Proteus sepsis after irradiation in the C57 parental mice.

Tumor

Five million live leukemia L1210 cells given i.p. were the tumor (antigenic) challenge in most experiments. Irradiated recipients were challenged with 50 million nonviable (UV-irradiated) cells because of their increased susceptibility to leukemia. This dose of killed tumor cells elicited the same degree of cellular immunity as the smaller number of live cells. The day of the challenge with tumor cells was designated “Day 0.”

Production of Hyperimmune Isoantibody

C57 mice were immunized with 8 to 10 weekly injections of 5 million L1210 cells i.p., following which Freund’s complete adjuvant was used to elicit antibody-rich ascites (4). As much as 10 ml of ascites were obtained from each mouse and were centrifuged free of cells. After titration of individual fluids, those with the highest titers of isohemagglutinins and cytotoxic antibody (q.v.) were pooled and used in a series of experiments. Antibody-rich ascitic fluid is termed “antibody” and was used in this study.
That end point was the last well-showing mat of agglutinated which the trays were shaken. Agglutination patterns were read Spectrazyme (Spectra Biologicals, East Brunswick, N. J.) and reciprocal of the dilution of serum present at the end point. It has been found macroscopically within 90 min. This method has been described in more detail elsewhere (17). It has been found that 25 ßl of washed 2% mouse erythrocytes were added, after 25/ul quantities of all reagents were used in microtrays. A concentration of 5 X 10^6 fresh L1210 cells/ml, a 1:3 dilution of guinea pig complement, and Fischer’s medium, without horse serum, as diluent were used in the assay. Cytotoxic antibodies in the pools of antibody-rich ascites were sufficient to cause 100% lysis of target cells in the 1st 4 to 5 wells.

Measurement of Remaining Tumor Cells

Irradiation Experiments. Mice were lethally irradiated in a 12-chambered Plexiglas cage with 800 R of whole-body irradiation, delivered by a Siemens 250-kv machine, with a 2-mm aluminum filter, at a distance of 70 cm (rate, 85.7 R/min). Spleen cells used for repopulation of recipient animals were obtained by gentle homogenization in a glass tissue grinder. These were washed twice in Fischer’s medium without horse serum and were resuspended with a 27-gauge needle. Aggregates were allowed to settle for 3 min before injection. Femoral bone marrow was obtained by flushing with 1 ml of medium, washing the cells, and resuspending as with spleen cells. All cells were injected i.v. within 4 hr after irradiation. Subsequent i.p. challenge with killed L1210 was accomplished 1 day later. Mice received tetracycline in their drinking water and were housed 1 to a cage to avoid infection. Since the number of cells per spleen was usually one-fifth to one-seventh of that found in other experiments, 5 X 10^6 spleen cells and 5 X 10^6 P-815 Y cells were incubated in the assay of cellular immunity to maintain the ratio of 100 spleen cells to 1 tumor cell, in a total volume of 1.0 ml.

RESULTS

Inhibition of Cellular Immunity by Isoantibody. Four injections of 0.1 ml of hyperimmune Ab administered on Days -4 to -1 before the injection of leukemia L1210 completely suppressed spleen cell-mediated immunity (Chart 1). Whereas controls given 0.9% NaCl solution, normal ascitic fluid, or hyperimmune Ab to 3rd-party (C3H) spleen cells.

The abbreviation used is: Ab, hyperimmune antibody-rich ascitic fluid against L1210.
Central Inhibition of Cellular Immunity

PRETREATMENT: DAY (-4) to (-1)

- New Ab
- Stored Ab
- Normal Ascites
- Saline
- Anti-C3H

Chart 1. Complete suppression of cellular immunity by pretreatment with 4 days of 0.1 ml of isoantibody. Abscissa, modes of in vivo treatment; ordinate, mean % lysis ± S.E. with the actual values shown above each bar. Numbers in parentheses here and in subsequent charts indicate the numbers of mice examined.

Chart 2. Comparison of pretreatment with a single 0.1-ml dose of Ab and posttumor treatment with 0.4 ml. Treatment before the tumor challenge was considerably more effective, as shown by the proportionately greater degree of suppression achieved by the lower dose.

Chart 3. Titer of isohemagglutinins 10 days after LI210 in Ab-treated mice and controls. All mice were C57 except as indicated. Note similar titers in all groups. Absence of isohemagglutinin titers in unchallenged recipients of stored Ab and of challenged BDF, recipients indicated de novo synthesis (see also Chart 4).

Chart 4. Decay of isohemagglutinin titers in the serum of mice given Ag. In each half of the chart, the first point is the original titer of the Ab before injection. Mean values from 2 studies are shown. Although passively administered "new" Ab (2 pools shown) by itself could have led to titers similar to those found in mice also given L1210, sequential study strongly suggested de novo synthesis. Stored Ab again did not lead to detectable serum titers on Day 10 unless animals were challenged.

them with tumor. These mice were ordinarily incapable of making a measurable response to L1210 by our assay procedure. No hemagglutinating or cytotoxic Ab activity was found in the serum of BDF, mice that received stored Ab even after challenge with L1210. These results in C57 and BDF, mice were interpreted to mean that the antibodies found in challenged C57 recipients were formed de novo in response to L1210 and were not simply derived from transferred stored Ab.

Further evidence for active antibody synthesis came from serial studies of titers of antibody in the serum of C57 mice given new Ab. Although hemagglutinins and cytotoxic antibodies were present in the serum of C57 recipients of new Ab 10 days after transfer regardless of challenge, 2 sequential studies of such animals revealed a rapid decay and a later reappearance of Ab indicative of de novo antibody production (Chart 4). Recipients of stored Ab showed a more rapid decay

immunosuppressive properties of the Ab preparation. Sorption with C57 or C3H spleen cells had no effect.

The importance of scheduling was emphasized by the finding (Chart 2) that a single 0.1-ml injection of Ab 1 day before LI210 was more effective than a 4-day course of 0.4 ml given after the antigen. In the latter group, only the mice treated with cytotoxic new Ab had a statistically significant reduction in cellular immunity. The pretreated mice all showed significant suppression compared to 0.9% NaCl solution-injected controls.

Ab obtained 15 days after a single immunization with 5 X 10⁶ L1210 cells with a hemagglutination titer of 3 to 4 did not suppress cellular immunity at any schedule.

Lack of Inhibition of Antibody Production. The inhibitory effect of Ab was limited to cellular immunity, with the formation of both isohemagglutinins and cytotoxic antibodies usually unaffected (Chart 3). No hemagglutinating or cytotoxic activity was detected in C57 mice 10 days after pretreatment with stored Ab unless they received an antigenic challenge. To help eliminate the remote possibility of a nonspecific restoration of activity and persistence of titer of stored Ab in vivo after challenge, we treated C57BL/6J X DBA/2JF₁ (BDF₁) mice with Ab and challenged...
of both hemagglutinating and cytotoxic antibody than with new Ab. Once again there was no titer of either kind of antibody 10 days after transfer of stored Ab unless mice were challenged with L1210.

Effect of Ab upon the Number of Tumor Cells. The number of L1210 cells was reduced considerably in mice treated with hyperimmune Ab before or after L1210 (Chart 5). The reduced number of tumor cells did not entirely explain the decrease in cellular immunity, however, since there were as few cells in the group treated after the tumor challenge as in those treated before challenge, the immune responses of which were considerably different (cf. Charts 1 and 2). Both new and stored Ab reduced the number of tumor cells to the same extent, suggesting either that the humoral immunity of the host participated in the removal of tumor or that an additional tumoricidal mechanism not involving cytotoxic antibody was implicated.

Injection of UV-killed tumor cells at a number approximating the minimal number of tumor cells remaining in the abdomen (10⁷), with or without pretreatment with Ab, revealed that Ab-treated mice consistently had a lower level of cellular immunity. Spleen cells from the latter group failed to lyse target cells [mean lysis, -4.7% ± 1.2 (S.E.)], whereas the injection of 10⁷ killed cells alone led to a mean lysis of 32.2% ± 9.1. Two further injections of killed cells on Days 2 and 4 partially overcame the effect of pretreatment with Ab, but cellular immunity in the Ab-treated group was still less than in controls (lysis of 41.0% ± 7.0 versus 69.3% ± 5.1, respectively). Each of these groups contained 6 mice. The number of tumor cells remaining in Ab-treated mice was thus shown to be sufficient to elicit immunity.

Evidence for a Central Effect of Ab. If the reduced number of tumor cells was not the entire explanation for the complete abolition of cellular immunity achieved, a mechanism immediately suggested was "afferent" inhibition by Ab, through the coating and blockade of antigenic sites. Yet despite the initial decrease in the levels of circulating Ab after L1210 challenge, suggesting such an absorptive effect of tumor cells, the unimpeded production of antibodies made a total blockade of antigenic sites highly unlikely. A more selective, and therefore perhaps central, inhibition of cellular immunity was suggested.

To avoid contact of Ab with antigenic tumor cells peripherally (i.e., distant from lymphoid organs), immunization by L1210 was accomplished in mice that had been lethally irradiated and then reconstituted with Ab-treated syngeneic spleen cells. For in vitro treatment of spleen cells 0.5 ml of new Ab was incubated with 10⁸ cells in a total volume of 2.5 ml at 25° for 60 min, after which the cells were washed twice and resuspended at a concentration of 5 x 10⁷ cells/ml. Two-tenths ml of the suspension (10⁷ cells) was injected i.v. into irradiated recipients. One million bone marrow cells were also injected in most experiments to increase survival. For in vivo treatment of donor spleens, 0.4 ml of Ab was given i.p. in 1 or 4 doses, allowing 48 hr between the last dose and the processing of the spleen cells. Spleen cells were washed twice and injected as above. No isohemagglutinins were detectable in the final wash fluid, nor were circulating (eluted) isohemagglutinins detectable in the serum of recipient mice before tumor challenge 1 day after transfer of the spleen cells. Fifty million UV-killed L1210 cells were injected i.p. 1 day after injection of in vitro- or in vivo-treated spleen cells. Mice were sacrificed 10 days after antigenic challenge. Mortality in all experiments to be described was less than 20%.

As illustrated in Charts 6 and 7, either in vitro or in vivo pretreatment of spleen cells with Ab was effective in suppressing the development of cellular immunity in the irradiated recipients. Although there was often no statistical difference between the number of cells in the spleen of Ab-treated mice and that in normal ascites-treated controls, the former was always lower. In vitro incubation of donor spleen cells with Ab achieved complete suppression of cellular immunity in mice repopulated only with spleen cells. Although bone marrow cells were injected only to increase survival, there was a significant (p < 0.001) partial repletion of immunity in recipients of such spleen cells and bone marrow cells together (Chart 6). The latter group was still considerably lower than immune controls, however. Mice receiving spleen cells from donors treated in vivo with Ab had a very low level of cellular immunity even when bone marrow cells were injected too, perhaps because the bone marrow cells used were from the same Ab-treated donors rather than normal mice (Chart 7).

If spleen cells preincubated in vitro with Ab were injected together with normal bone marrow cells and were allowed to repopulate recipients for 12 days before challenge with L1210, there was still a significant deficit in the cellular immunity achieved: 60.9 ± 4.9 versus 92.0 ± 2.1 in mice receiving spleen cells incubated with normal ascites. There were 5 mice examined in each of these groups. Spleens in both of these groups were of normal adult size, containing approximately 7 x 10⁷ cells.

Although transfer of some free Ab with the spleen cells was not entirely ruled out, the profundity of suppression in recipients even after delayed challenge with antigen appeared to militate against afferent (peripheral) blockade.

Effect of Fractions of Ab upon Cellular Immunity. IgM and IgG fractions of Ab were obtained by Sephadex G-200 column chromatography (2.5 x 40 cm; 1.2-ml sample). IgM was eluted pure in Peak 1; there was some contamination of IgG by IgA.
immunity when normal bone marrow was injected concomitantly. Complete suppression was achieved, with partial restoration of transfer to lethally irradiated recipients and challenge with L1210. Bone marrow from the same donors was also given. A significant inhibition of cellular immunity was again achieved with L1210 challenge. A quantitative in vitro assay for the tumoricidal activity of spleen cells has made it possible to determine that the reaction of the host to leukemia L1210 was abolished by pretreatment with Ab even when the leukemia did not grow progressively. We have considered this phenomenon to be enhancement as defined in its broadest sense, i.e., Ab-mediated suppression of the cellular immune response to foreign tissue.

DISCUSSION

True enhancement of the growth of murine leukemias has been achieved only rarely, usually by drastically altering the ratio of antibody to cells (8). With an alternative method Batchelor (5) and Chard et al. (11) found that Fab fragments from the IgG fraction of hyperimmune antibody were able to enhance EL-4 leukemia. Such fragments lacked cytotoxicity, since the complement-fixing Fc portion was absent. More commonly the cytotoxic effects of antibody have been most prominent because of the densely packed antigens on the surface of the well-dispersed, easily accessible ascites leukemia cells (14, 16, 19, 20). A quantitative in vitro assay for the tumoricidal activity of spleen cells has made it possible to determine that the reaction of the host to leukemia L1210 was abolished by pretreatment with Ab even when the leukemia did not grow progressively. We have considered this phenomenon to be enhancement as defined in its broadest sense, i.e., Ab-mediated suppression of the cellular immune response to foreign tissue.

Brent and Medawar (9) suggested that isoantibody might act upon host cells in a central form of enhancement, in addition to any action it might have directly upon the antigen. While Moller (20–22) has convincingly shown that afferent inhibition of immunity, i.e., that caused by blockade of antigenic sites before sensitization of lymphocytes, is 1 mechanism for enhancement of tumor growth, nevertheless there seems to be firm evidence for both central and afferent forms too. For example, sensitized lymphocytes were blocked from killing tumor cells in vitro in the presence of antibody (15), ostensibly an afferent inhibition. The best direct evidence for enhancement mediated at a central level may be the experiments of Takasugi and Hildebrand (26, 27), in which they demonstrated that treating with antibody to Sarcoma I, or its IgG\(\gamma_1\) or IgG\(\gamma_2\) fraction, inhibited cellular immunity to the tumor. Ab synthesis was unimpaired though slightly delayed. Reactive lymphocytosis to the tumor was diminished in antibody-treated mice, an effect not influenced by the presence of the tumor and thus not due to an afferent or efferent blockade. Both IgG and IgM antibody were cytotoxic to the tumor, but the latter did not enhance its growth, indicating that attachment to the tumor was not sufficient to explain the enhancement.

A strong argument in favor of a central mechanism of immunosuppression by antibody is that it requires only very small amounts. Crowle and Hu (12) produced suppression of cellular immunity to ovalbumin with as little as 1.2 μl of antibody given during the period of induction of immunity.
Delayed sensitivity was more completely suppressed at all schedules of administration of antibody than was immediate sensitivity. Ryder and Schwartz (24), in studying the regulation of antibody synthesis, found that passively administered antibody appeared to inhibit the processing of sheep RBC by macrophages. In their experiments, the small quantities of antibody that suppressed antibody synthesis were often given weeks before the antigen and were clearly incapable of blocking all antigenic sites. Any Ab that might have been transferred or eluted, undetected, from transferred spleen cells in our irradiated recipients also seems unlikely to have been sufficient to block L1210 afferently.

A central effect upon lymphocytes producing antibody to sheep RBC in rats was suggested to Rowley and Fitch (23) by prolonged effects of administered antibody, and irradiation-repopulation experiments were performed to provide definitive proof. They too found that pretreatment in vitro of rat spleen cells with isoantibody made those cells less effective immunologically upon challenge in the irradiated host. Although this sort of experiment has not always been reproduced successfully (24), our similarly designed experiments repeatedly provided the clearest evidence for an effect of Ab upon host cells. The partial repletion by bone marrow cells of immunity suppressed by exposure of spleen cells to Ab in perplexing if thymus-derived lymphocytes are responsible for cellular immunity. The in vivo target of Ab may conceivably be (bone marrow-derived) macrophages for which the Ab is cytophilic, with a consequent effect upon lymphocytes, but this remains to be proved.

The subcellular basis for central inhibition is also obscure and involves the question of whether antigen-antibody complexes are involved. Recent in vitro experiments (13) in which low-zone tolerance required small amounts of antibody as well as antigen bring the phenomenon of tolerance closer to enhancement, suggesting that immune complexes may be crucial in both. A similar involvement of antigen-antibody complexes has been indicated in the feedback inhibition of antibody synthesis (25). Experiments with irradiation and adoptive transfer of Ab-treated cells here ostensibly did not require antigen at the earliest stage of Ab’s interaction with the cells, but the suppression might have been initiated only after antigen was also present. We are currently varying the timing of antigen after Ab to elucidate the mechanism further.

Although we have emphasized the immunologically more interesting suppression of cellular immunity induced by Ab, treatment with Ab after challenge with L1210 had very little effect upon either cellular immunity or antibody synthesis and considerably reduced the large number of tumor cells injected. Moreover, the ability of mice completely suppressed by Ab to reject the tumor and thus survive reemphasizes the relative unimportance of lymphoid cell-mediated immunity in the rejection of murine leukemia, as opposed to the role of antibody (1). A form of cellular immunity mediated by peritoneal macrophages may be of preeminent importance in the rejection of ascites tumors (2, 3), but there cytophilic antibodies are also involved. Administered Ab also has immunosuppressive effects upon macrophage-mediated cellular immunity, which will be discussed in a subsequent paper (18).

Finally, one may postulate that antibody normally synthesized during the immune response to murine leukemia acts as an endogenous regulation of concomitant cellular immunity. Such a concept may be useful in explaining the balance normally achieved in the successful rejection of transplantable allogeneic tumors, where reciprocal changes in humoral antibodies and cellular immunity are known to occur at the time of rejection. This form of “feedback” regulation of cellular immunity may well be a more general phenomenon in the immune response than in the system we have examined, as has been postulated by several others after examination of the response to different antigens (5, 12, 26, 27).

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