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A NEW ATIGEN IN LEUKEMIA L1210 SUBLINE (RESISTANT TO METHYLGLYOXAL-BIS-GUANYLHYDRAZONE)

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INTRODUCTION

In the DBA/2Ha-DD mouse, leukemia L1210 grows progressively and eventually results in the death of all animals inoculated with 10~100 cells or less 1,2). Indeed this leukemia is considered to be specific for the DBA/2 strain, because it does not grow at such low inocula in any of the other mouse strains tested. On the other hand, an immunological response sufficient to cope with a challenge of $10^3$ leukemic cells became effective in DBA/2 mice previously inoculated with $10^6$ cells and cured with selective chemotherapeutic treatments 3,4).

Initial investigations 2,4) showed that the greater therapeutic responses in nonirradiated mice were attributed to a greater effectiveness of host defenses against the resistant cells than against the parent cells since no difference in therapeutic effects was noted in preirradiated animals. In the absence of chemotherapy, survival of mice from 3 DBA/2 lines was longer after the i.p. inoculation of resistant leukemic cells than after inoculation of sensitive cells in corresponding numbers, when $10^5$ cells or less were given 2). These findings suggested that the L1210 subline which is resistant to methylglyoxal-bis-guanylhydrazone is more immunogenic than the parent leukemia in the mouse lines tested or is more sensitive than the latter to the immunological response of these mice.

The present study was carried out in an attempt to clarify whether the resistant subline is more immunogenic than the parent line or solely more sensitive than the latter to the host response and also to determine whether the possibly greater immunogenicity of the resistant subline is due to the presence of the resistant cells or of different antigens. To this end, differences of antibody binding to antigenic sites actually present on the 2 cell types were investigated by means of a paired-label antibody technique. These results have been reported in a preliminary communication 5,6).

MATERIALS AND METHODS

Animals and cells: The animals used were 2~3 months old DBA/2Ha-DD female mice weighing 20~25 g. They were obtained from the breeding colony of Roswell Park Memorial Institute.
The leukemia L1210 used in this study was presented from Dr. A. Goldin, National Cancer Institute, in 1957 and thereafter was transferred every 6~7 days in female DBA/2Ha-DD mice by the i.p. inoculation of $10^6$ ascites cells. The subline resistant to methylglyoxal-bis-guanylhydrazone (L1210/CH$_3$-G) was developed in Roswell Park Memorial Inst. in 1966 and was thereafter transferred in a manner similar to that used with L1210, except that the host mice were treated i.p. once daily for 4~6 days with methylglyoxal-bis-guanylhydrazone, 50 mg/kg.

The target leukemic cells used in the in vitro antibody-binding reactions were obtained from the peritoneal cavity of mice that had been inoculated with $10^6$ cells 3 days before sacrifice. The cells were washed once with RPMI 1640 medium, separated from red blood cells by Ficoll gradient centrifugation, and finally washed 3 times with RPMI 1640 medium. Normal spleen cell suspensions were obtained by mechanical dissociation through a stainless steel wire mesh and were treated in the same way as were the leukemic cell suspensions. Suspensions of cells from spontaneous mammary tumors of the DBA/2Ha-DD mouse were obtained by gentle stirring of 1~2 mm$^3$ tumor pieces in ice-cold Earle's solution 6 or 30 min, without the addition of enzymes. After this stirring the suspension was filtered through 3 layers of gauzes, and the cells were washed 3 times with RPMI 1640 medium. The viability of the target cells was determined by trypan blue staining.

Preparation and iodination of y-globulins: One million L1210 or L1210/CH$_3$-G cells were inoculated i.p. into DBA/2Ha-DD mice. Arabinosylcytosine was injected i.p. at the dose of 10 mg/kg/day for 6 consecutive days, starting the day after that of leukemia inoculation. Animals surviving on day 50 were challenged with $10^5$ live leukemic cells and were rechallenged at 2-week intervals with successively increasing numbers of cells ($10^4$, $10^5$, $10^6$ and $10^7$). Those sera were obtained 2 weeks after the last challenge and were stored at $-70^\circ$C before use.

Two ml of serum were dialyzed overnight against 0.02 M PBS (pH 8.0) and then centrifuged for 15 min, at 4$^\circ$ and 3000 rpm. The y-globulin was obtained from the supernatant by DEAE cellulose column fractionation at pH 8, after suitable concentration. The y-globulin solutions were kept at $-20^\circ$ and were used within 2~3 months after fractionation.

Gamma-globulin from antiserum was labeled with $^{125}$I and y-globulin from normal serum was labeled with $^{131}$I by the chloramine-T method. The iodinated preparation contained 1~2 mCi of label per mg of protein and was kept frozen. The $^{125}$I-labeled immune y-globulin preparation was mixed with the $^{131}$I-labeled normal y-globulin preparation at an appropriate ratio, usually 1 : 1 with respect to protein content. The day before the specific uptake test, the paired-label mixture (P.M.) was absorbed with 5~10~10$^8$ normal spleen cells from female DBA/2Ha-DD mice at 37$^\circ$ for 1 hr. Immediately before use, P.M. was centrifuged for 60 min at 4$^\circ$ and 40,000 rpm in an ultracentrifuge.

For the main test, 0.5 ml of P.M. was placed into an 8-ml tube, and 0.1 ml of the desired target cell suspension in RPMI 1640 medium ($10~10^6$ cells) was added. After 60 min of incubation at 37$^\circ$ with gentle shaking, the cells were washed twice with RPMI 1640 medium and transferred into new tubes. The radioactivities in pellets obtained by centrifugation at 3,000 rpm for 20 min were measured in a 2-channel y-ray spectrometer.

The specific antibody uptake in the target cells was calculated according to the following
formula:

\[
\text{Specific uptake} = \frac{(^{125}\text{I} \text{ cpm on leukemia cells}) - (^{131}\text{I} \text{ cpm on leukemia cells})}{^{125}\text{I} \text{ cpm/ng of immune } \gamma\text{-globulin}} \\
\text{Ro} = \frac{^{125}\text{I} \text{ cpm on normal cells}}{^{131}\text{I} \text{ cpm on normal cells}}
\]

The detail of this method was described elsewhere \(^8\).

**RESULTS**

The results of typical in vitro uptake of anti-L1210 and anti-L1210/CH\(_3\)-G \(\gamma\)-globulin by L1210 and L1210/CH\(_3\)-G cells are shown in Figure 1. In this experiment, the undiluted paired-label reagents were incubated with 5 or 10 million cells.

**Fig. 1.** Specific uptake of immune \(\gamma\)-globulin by L1210 or L1210/CH\(_3\)-G cells as a function of the number of reacting cells.

![Graph showing specific uptake](image)

Left: results obtained with the paired-label mixture containing \(^{125}\text{I}\)-labeled anti-L1210 \(\gamma\)-globulin. Right: results obtained with the paired-label mixture containing \(^{125}\text{I}\)-labeled anti-L1210/CH\(_3\)-G \(\gamma\)-globulin. SMT: spontaneous mammary tumor cell. Bars: average value of 4 determinations and vertical line is standard deviation.

As is shown on the left part of the figure, the specific uptake of anti-L1210 \(\gamma\)-globulin on L1210 cells was about 4 times that on L1210/CH\(_3\)-G cells, regardless of the number of cells.
incubated. Conversely, as is shown on the right part of the figure, the uptake of anti-L1210/CH₃-G γ-globulin was higher on L1210/CH₃-G than on L1210 cells. In this case, the difference was about 10-fold. The specific uptake of this antibody to cells from a spontaneous mammary tumor of DBA/2Ha-DD mouse was nil. Although the specific uptake of each antibody on the cross-reacting leukemic cells was of the same order of magnitude, the specific uptake of anti-L1210/CH₃-G γ-globulin on L1210/CH₃-G cells was about twice that of anti-L1210 γ-globulin on L1210 cells.

The specific uptake of antibody by the leukemic cells was also tested as a function of serial dilutions of the paired-label reagent. The results obtained are summarized in figure 2. Anti-L1210 γ-globulin was specifically taken up by L1210 more than by L1210/CH₃-G cells. Conversely, anti-L1210/CH₃-G γ-globulin was taken up by L1210/CH₃-G more than by L1210 cells. The ratio between specific uptakes by the 2 cell types was constant, regardless of the dilution of the paired-label mixture, and was smaller for anti-L1210 γ-globulin than for anti-L1210/CH₃-G γ-globulin.

Fig. 2. Specific uptake of immune γ-globulin dilutions by L1210 or L1210/CH₃-G cells.

The results obtained as shown in figure 1 and 2, respectively, are consistent in suggesting that γ-globulin anti-L1210/CH₃-G contains more specific antibody than γ-globulin anti-L1210, and that, above a relatively constant level of cross-reactivity, specificities are different between the 2 cell types.

The distinct specificities of γ-globulin anti-L1210/CH₃-G were verified further by pre-
absorbing 10～50 ng of various types of unlabeled γ-globulin on 10 million target L1210/CH₃-G cells for 60 min and then reacting the paired-label mixture containing 10 ng γ-globulin anti-L1210/CH₃-G. The results of this "blocking" experiment, which are shown in figure 3, indicated that preabsorption with γ-globulin from normal mice or from mice treated with DBA/2Ha-DD liver homogenates had no influence on the subsequent specific uptake of anti-L1210/CH₃-G γ-globulin as same as that with RPMI 1640 for a control. In contrast, preabsorption with anti-L1210 γ-globulin reduced the subsequent specific uptake of anti-L1210/CH₃-G γ-globulin by 43～63%, whereas preabsorption with anti-L1210/CH₃-G γ-globulin blocked the subsequent specific uptake of this γ-globulin by 61～74%.

**Fig. 3.** Effect of preliminary incubation with various amounts of unlabeled IgG on specific uptake of 10 ng labeled anti L1210/CH₃-G antibody.

Corresponding weights (ng) of unlabeled IgG on the axis of abscissa were used for preabsorption as a blocking test. RPMI 1640; without any γ-globulin for a control.

The possibility that L1210/CH₃-G has antigenic specificities different from those of L1210 was investigated further in next experiment which the effects of repeated prior absorption of antibody with L1210 or L1210/CH₃-G cells on the subsequent specific uptake of this antibody by homologous cells were measured. The results obtained are shown in figure 4. After 1 absorptions of anti-L1210 antibody with either L1210 or L1210/CH₃-G cells, the slight difference in residual specific uptake by L1210 cells was not significant. After 2～4 serial absorptions, no difference in residual specific uptake by L1210 cells was found. In contrast, as shown by the divergence of the corresponding curves after 3 subsequent absorptions, anti-L1210/CH₃-G
Fig. 4. Residual specific uptake of immune γ-globulin by homologous cells after sequential absorption with L1210 or L1210/CH₃-G cells.

The initial specific uptakes of non-absorbed γ-globulins by homologous cells (not shown) was 5.5 ng × 10³ for anti-L1210 γ-globulin and 16.2 ng × 10³ for anti-L1210/CH₃-G. γ-globulin antibody was absorbed more completely by L1210/CH₃-G than by L1210 cells, at least within the scope of the 10 repeated absorptions. The fact that no significant differences in residual specific uptake by L1210/CH₃-G cells was noted after 1–2 absorptions with either L1210 or L1210/CH₃-G cells is probably related to the fact that the anti-L1210/CH₃-G immune γ-globulin contains a relatively large amount of antibody. Therefore, it is conceivable that the 10 million cells used for each absorption were not sufficient to put in evidence differential absorption until most of the cross-reacting specificities were taken up. These data further support the conclusion that more antibody is formed after immunization with L1210/CH₃-G than with L1210 cells and that the antigenic specificities of the resistant cells are different from those of the parent cells.

DISCUSSION

The previous paper² demonstrated that L1210 and L1210/CH₃-G cells were immunogenic in the DBA/2Ha-DD mice compared with other strains and L1210/CH₃-G cells were more immunogenic than L1210 cells in this mouse. Moreover, in this investigation, it was found that the specific uptake of anti-L1210/CH₃-G γ-globulin by L1210/CH₃-G cells was at least 2 times as high as that of anti-L1210 γ-globulin by L1210 cells. This greater specific uptake of γ-globulin anti-L1210/CH₃-G by L1210/CH₃-G cells can not be ascribed solely to the presence of more antigen or antibody-binding sites on the resistant cells, because the specific uptake
of anti-L1210 γ-globulin was greater on L1210 cells than on L1210/CH3-G cells (figure 1). Therefore, the antigenic specificities on the 2 cell lines must be different, at least in part. This conclusion is supported further by the observation that 10 repeated absorptions of anti-L1210/CH3-G cells γ-globulin do not reduce the residual specific uptake of the absorbed γ-globulin by L1210/CH3-G cells as completely as 10 absorptions with homologous L1210/CH3-G cells do. On the other hand, in figure 1 are those responsible for the relatively low level of cross-reactivity between the 2 cell lines, those present only on L1210 cells, and those present only on L1210/CH3-G cells. If only 2 groups of antigens exist on these cells, one would have to assume that antigenic binding sites characteristic of L1210 cells are available on L1210/CH3-G cells in a quantity comparable to that of antigenic binding sites characteristic of L1210/CH3-G cells available on L1210 cells. The fact that no specific uptake of anti-L1210/CH3-G γ-globulin was seen with spontaneous mammary tumor cells from DBA/2Ha-DD mouse, also, substantiates the specificity of this γ-globulin.

The initial observation2,4), that low doses of arabinosylcytosine cause a higher incidence of 50 day cures among DBA/2Ha-DD mice bearing the resistant subline than among those bearing L1210 and this difference was not seen in preirradiated animals, have been confirmed and extended in more recent studies. These data showed, that is, that DBA/2Ha-DD mice without a chemotherapy survived longer after the inoculation of 10⁵ or less L1210/CH3-G cells than after the inoculation of corresponding numbers of L1210 cells. Moreover, a significantly higher incidence of 50 day cures was obtained after selective treatments with 4 different drugs among the L1210/CH3-G bearing mice than among the L1210 bearing ones5). In both experiments, no difference in survival was found if the animal had been given a total-body X-ray irradiation the day prior to leukemia inoculation. Therefore, a question arose whether the mentioned differences between L1210 and L1210/CH3-G were related to a greater sensitivity of L1210/CH3-G cells to the host response or to a greater immunogenicity of these cells leading to a more effective host response. Although the results of the present study can not exclude the possibility that L1210/CH3-G cells are more sensitive than L1210 cells to immunotoxicity, these clearly demonstrate that L1210/CH3-G cells are more immunogenic than L1210 cells in the mouse used. The data reported herein are related only to measurements of antibody binding sites and do not cast any light on possible differences in cytotoxic antibodies against these 2 cell types, but these data are quite consistant and in parallel with the survival obtained in the animal1,2).

The L1210/CH3-G cells developed in Goldin laboratory (NIH) in 1966 were transferred every weeks into DBA/2Ha-DD mice treated i.p. with methylglyoxal-bis-guanilhydrazone as a maintainant transplantation. It was recently reported that some anti-cancer drugs arose the DNA- or chromosome-leakage of target tumor cells. Although the genetic or somatic changes of L1210/CH3-G cells after 185 transplant generations were possibly thought, no chromosome difference between L1210 and L1210/CH3-G cells was found (unpublished data).

It is possible that cell populations with increased immunogenicity may arise during treatment with a drug with immunosuppressive effects leading to a decreased immunoselection. Consistent with this hypothesis is the fact that methylglyoxal-bis-guanylhydrazone inhibited host defenses in mice. Goldin et al.9,10,11) and Hutchison et al.12) reported that the antigenicity of L1210 sublines with a increased immunogenicity were indeed resistant to drugs. The appearance
of increased antigenicity of L1210/CH3-G cells would appear to be attributable to the expression of new antigen as a result of the development of resistance. Obviously, further experiment will be required to prove that the validity of this idea can be assessed and that the basis for the development of more antigenic-resistant leukemia lines can be understood. Finally, the best immun chemotherapy which is considered not only drug function but also tumor immunity and host-defense reaction will be successful.

SUMMARY

Using paired label radio-antibody method which γ-globulin from antiserum was labeled with $^{125}$I and γ-globulin from normal serum was labeled with $^{131}$I, the following conclusions had been gotten:

1). Both L1210 and the resistant subline to methylglyoxal-bis-guanylhydrazone (L1210/CH3-G) cells are immunogenic in vitro and elicit a demonstrable humoral antibody response in the DBA/2Ha-DD mice.

2). The resistant subline L1210/CH3-G cells is more immunogenic than L1210 cells in DBA/2Ha-DD mice.

3). The antigenic specificity of L1210 is different from that of L1210/CH3-G cells, although both cell lines share some common antigens.

4). Therefore, the data support the hypothesis that new antigen(s) have been produced in drug-resistant sublines of leukemia L1210.

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