Role of Cellular Antigens in Humoral and Cell-Mediated Immunity as Measured *in Vitro*[1] (37717)

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The relative importance of cell-mediated immunity in effecting tumor allograft rejection, as compared to the weak or even enhancing effect of the humoral response, has been well-documented in the case of solid tumors (1). With leukemia allografts, it is generally assumed that cytotoxic antibodies have a determinant role in rejection (2).

Multiple antigenic specificities are expressed on the plasma membrane of nucleated cells (3, 4). It is reasonable to expect, therefore, that cell-mediated immune responses elicited by tumor cells be directed against more than one specificity, in analogy to the humoral response (2). *In vitro* methods are now available to measure by the same basic test the complement-independent (C'ICC) and complement-dependent (C'DCC) cellular cytotoxicity against nucleated target cells. Because of the specificity of both C'ICC, which is thought to measure a T-cell response (5), and C'DCC, which measures a cytotoxic antibody response (6), it is possible to evaluate *in vitro* the relative role of the various alloantigenic specificities expressed on a tumor cell in eliciting cellular and humoral immunity *in vivo*. The present initial study was carried out to provide an example of the type of information that can be obtained by the concurrent use of C'ICC and C'DCC in appropriate graft–host systems. The factors determining the type of immune responses elicited by a given polyantigenic system were investigated using leukemia EL4 cells as immunogen; DBA/2J, C3Hf/HeHa, and AKR mice as responders; and EL4 cells, C3Hf/HeHa thymocytes, and L1210 cells as targets for the *in vitro* tests.

The data obtained indicated that a humoral response can be elicited by any of the antigenic specificities examined, whereas a cellular response could be demonstrated against H-2 private antigen but not against H-2 public nor θ antigens.

Materials and Methods. Animals. Female C57BL/6Ja, DBA/2HaDD, C3Hf/HeHa, and AKR mice were obtained from the breeding colony of this institute. Female DBA/2J mice were purchased from the Jackson Laboratory, Bar Harbor, ME. The animals were used when 2–4 months old and were maintained on Teklad Mouse Breeders Chow and water *ad libitum*.

Nucleated cells used as antigen or target. Leukemias L1210 (7) and EL4 (8) have been transplanted at weekly intervals in DBA/2HaDD and C57BL/6Ja mice, respectively. For the purpose of this study, it will be assumed that EL4 cells express three major sets of antigenic specificities, one of which is shared by L1210 cells (Table I). This assumption is based on the results of *in vitro* immune cytolysis experiments carried out in this laboratory and published reports by others (9). L1210 cells are considered to be

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TABLE I. Antigens Expressed on Target Cells.

Antigens[a]	Target cells		
	EL4	L1210	C3H thymus cells
H-2 "Private"	H-2^b	H-2^d	H-2^k
H-2 "Public"	"C"	"C"	Not "C"
θ	θC3H	None	θC3H

[a] "C" represents common antigen(s) shared by C57BL and DBA/2 (therefore by EL4 and L1210). According to the H-2 chart (10), they are most probably the H-2^b and H-2^d public antigens coded for by the D-region of the H-2 locus. The remaining antigens of EL4 were not considered during this study.

H-2^d because this is a DBA/2 leukemia; likewise, EL4 is H-2^b by virtue of its C57BL/6 origin. Table II shows the serological evidence for the presence of the θC3H antigen on EL4 and the lack of expression of this antigen by L1210. The anti-C3H thymus and anti-EL4 sera have the same distribution of toxicity for EL4 cells and θC3H-bearing thymus cells; evidence for specificities other than θ was not considered since it is not relevant within the scope of this study.

The common antigen shared by EL4 and L1210 and called here "C" is illustrated by the fact that antisera from C3H or AKR toxic for one was also toxic for the other, and that this toxicity could be absorbed by either C57BL or DBA/2 normal cells (data not shown). Although no attempt has been made to determine whether this (or these) antigen(s) belongs to H-2 or represents minor histocompatibility antigens, it is reasonable to assume from the H-2 chart (10) that "C" represents essentially a group of H-2 public antigens, common to C57 and DBA/2 mice, coded by the D region (antigens Nos. 6, 27, 28, 29).

In summary, L1210 cells are considered to be H-2^d positive, θC3H negative, and to have an antigen also present on EL4, here called "C". EL4 cells are considered to be H-2^b, θC3H, and "C" positive. C3H thymus cells will be used here as representatives of the θC3H specificity. This is obviously an oversimplification justifiable only operationally within the scope of this study.

Immunization procedure. Unless otherwise specified, mice were immunized by a single ip injection of 1–5 $\times$ 10^7 nucleated cells suspended in 0.1–0.2 ml of saline.

TABLE II. Evidence for the Presence of θC3H Antigen on EL4 Cells.

Antiserum	Target cells[a]									
	C57Bl/6			DBA/2J			AKR		C3Hf/HeHa	
	EL4	BM	T	L1210	BM	T	BM	T	BM	T
AKR anti-C3H thymus[b]	71[c]	23	85	30	27	73	20	40	13	80
C3H anti-AKR thymus[c]	12	34	41	41	31	40	40	86	16	28
AKR anti-EL4[d]	78	47	85	40	26	78	15	13	13	80

[a] BM = bone marrow cells; T = thymus cells.

[b] The AKR anti-C3H thymus cell serum used here was collected 6 days after one ip injection of 3 $\times$ 10^7 C3H thymus cells. The presence of an anti-LyA_1 as a contaminant has not been ruled out but is irrelevant against EL4 or C57 thymus cells which are LyA_2 (9); it is apparent that this antiserum also contains an autoantibody as shown by 40% cytotoxicity against AKR thymus cells.

[c] The C3H anti-AKR thymus cell serum used here was collected 14 days after one ip injection of 3 $\times$ 10^7 AKR thymus cells. The presence of anti-LyA_2 antibodies was not ruled out.

[d] The AKR anti-EL4 serum was collected 7 days after the ip inoculation of 5 $\times$ 10^7 EL4 cells. It should be noted that this serum is as cytotoxic for θC3H positive thymus cells of DBA/2, C3H, and C57BL/6 as it is for EL4 cells.

[e] Tests were performed as follows: Aliquots of 0.1 ml of a 1:32 dilution of each antiserum were incubated for 30 min with 1 $\times$ 10^5 ^{51}Cr-labeled target cells in 0.1 ml of RPMI 1640 medium; 0.4-ml aliquots of 1:32 rabbit serum as a source of complement were added for 30 min. Values represent specific ^{51}Cr release.

Test procedure. Immune effectors as well as target cells were collected and prepared as described previously (6). Target cells were labeled with chromium 51 (sodium chromate-51, Amersham Searle 1 mCi/2–7 μg Cr/ml) according to the method of Sanderson, slightly modified (6). Cell-mediated immunity, called here complement-independent cellular cytotoxicity, complement-dependent cellular cytotoxicity, and serum antibody (SA) were all measured by the chromium-51 release assay (6).

Spleen cells were used as effector cells for *in vitro* cytotoxicity tests at a ratio of 100 effector cells:1 target cell. Complement was provided as fresh frozen rabbit serum and was used at a 1/32 dilution which had less than 10% cytotoxicity for the cells used. All incubations were performed in RPMI 1640 medium supplemented with 10% fetal calf serum.

The C'ICC response was measured after 4 hr of incubation. For the C'DCC response, incubation was carried out for 1 hr after which complement was added and incubation continued for 1 more hr (6). Serum antibody was measured after 0.5-hr incubation of serum and target cells and a further 0.5 hr after the addition of complement. All incubations were performed in a New Brunswick Gyrotatory shaker bath at 37° under humidified 10% CO_2 and air mixture with mild circulatory agitation.

Results. A humoral response against the antigenic specificities considered by the strains of mice used was predicted as shown in Table III. Within the scope of this prediction, the specificities against which C'ICC, C'DCC, and SA were directed were analyzed, as were the relationships among them.

TABLE III. Expected Serological Specificities of the Anti-EL4 Immune Response in the Strains of Mice Used.

	Expected serological specificities		
Strain of mice	Anti-H-2^b	Anti-"C"	Anti-θC3H
C3Hf/HeHa	+	+	—
DBA/2J	+	—	—
AKR	+	+	+

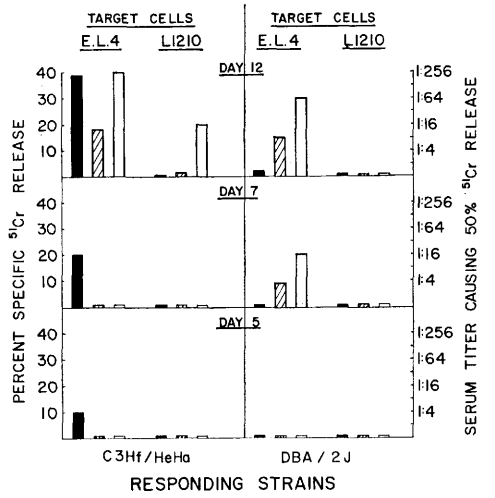

FIG. 1. Comparison of the immune responses of female C3Hf/HeHa and DBA/2J mice to the C57BL/6 lymphoma EL4. *In vitro* cytotoxic tests were performed using both EL4 and L1210 (DBA/2 strain leukemia) as target cells. Responding mice were given a single ip inoculation of 3×10^7 EL4 cells on day 0. The mice were then sacrificed on the days indicated. C'ICC (5) was assayed by incubating spleen cells and labeled target cells at a 100:1 ratio for 4 hr (solid bars). C'DCC (6) activity was assayed by incubating spleen cells and target cells at 100:1 ratio for 1 hr followed by the addition of rabbit complement and further incubation for 1 hr (hatched bars). SA (6) was assayed by incubating target cells and the appropriate serum dilutions for 1 hr followed by the addition of rabbit complement and further incubation for 1 hr (empty bars). C'ICC and C'DCC values are expressed as percent specific release (left ordinate). SA values are expressed as the highest serum titer causing 50% specific release (right ordinate). Five mice were in each group for each experiment, and the corresponding spleens were pooled for the assays. Each experiment was repeated a minimum of three times; thus, each bar represents the average values from a minimum of 15 mice.

C3H and DBA/2 responses against EL4 (H-2^b, "C", θC3H) measured in vitro *using EL4 and L1210 cells (specification for "C") as targets.* As early as day 5 after immunization with EL4 cells, C3Hf/HeHa mice exhibited a C'ICC response against EL4 cells, but not a C'DCC or SA (Fig. 1). The C'ICC response was specific against EL4 with no detectable effect against L1210 ("C" antigen). In the same mice, by day 7, the C'ICC

response was greater, but no C'DCC or SA response was noted as yet. On day 12, both C'DCC and C'ICC responses could be detected. In the presence of complement, the C3H antiserum was cytotoxic not only for EL4 (1:256) but also for L1210 (1:16) cells. Thus, it would appear that the "C" antigen elicits primarily a humoral response.

By day 5, DBA/2J mice did not exhibit any measurable C'ICC, C'DCC, or SA response against EL4 cells. By day 7, no C'ICC was detected as yet; however, a C'DCC was measurable, and the serum was cytotoxic for EL4 (1/16). By day 12, a small C'ICC response could be measured (4–7%). The C'DCC response against EL4 was moderate (15%), and the serum was cytotoxic (1/64). Data not shown indicated that the C'ICC response against EL4 became stronger after day 12 in DBA/2J mice, reaching by day 16 almost the level seen at earlier days in C3Hf/HeHa mice. As expected, no response could be detected against the "C" antigen in L1210 (DBA/2 leukemia).

A dichotomy was seen between the two types of early primary immune response against EL4 in the two strains of mice. In C3H mice which recognized at least two different antigens (H-2^b and "C") present on EL4, the C'ICC response was rapid but the humoral response notably delayed. The part of the humoral response that was directed against "C" did not seem to impair the C'ICC directed against H-2^b on EL4 cells, while no C'ICC could be detected against "C" on L1210 cells. Thus, in this system, no appreciable inhibition of the C'ICC anti-H-2^b private antigens occurred in the presence of the humoral response to the D-end public antigens. DBA/2J mice had a rapid humoral response to EL4 and a delayed C'ICC; the humoral response was rapid in spite of the fact that it was presumably elicited only by part of the H-2 antigens (H-2^b "private", namely H-2 minus "C" or the D-end "public" antigens). It should be stressed that C3H mice die after the ip inoculation of 5×10^7 EL4 cells around days 12–16 (data not shown), consistently with results previously obtained with leukemia L1210 (6). DBA/2J, on the other hand, always rejected a 5×10^7-EL4 cells inoculum.

C3H and AKR responses against EL4 (H-2^b, "C", θC3H) measured in vitro *using EL4, L1210 (specification for "C"), and C3H thymus cells (specification for θC3H) as targets.* The responses of C3Hf/HeHa mice to EL4, as measured *in vitro* using EL4 and L1210 cells (Fig. 2), were consistent with results obtained in the preceding group of experiments (see Fig. 1). In no case could the responses of C3H mice to EL4 be detected using C3H thymus cells as targets (Fig. 2).

AKR mice, by day 5, exhibited a weak C'ICC (7–10%) specific for EL4. Almost no C'DCC was detected against EL4 and L1210, but a very strong C'DCC was seen against C3H thymus cells (56%). Serum antibody was present against EL4 (1/64) and C3H thymus (1/1280), but not against L1210 cells. Therefore, one can assume that most of the early serum activity seen against EL4 in AKR mice is directed against the θC3H specificity present on EL4 beyond and above a possible response to H-2^b.

By day 7 after immunization, AKR mice had a C'ICC specific for EL4 (no effect on L1210 or C3H thymus). The C'DCC could be measured against EL4 (32%), L1210 (4.5%), and C3H thymus cells (53%); the SA affected EL4 (1/320), L1210 (1/80), and C3H thymus cells (1/1280). Thus, at this time point, both anti-"C" and anti-θC3H humoral responses could be detected separately from a possible response against H-2^b.

By day 14, AKR mice had a small C'ICC against EL4 and none against L1210 or C3H thymus; a C'DCC could still be measured against thymus but not against EL4 cells. The SA affected C3H (1/4096), EL4 (1/256), and L1210 cells (1/64). This activity could be entirely absorbed by C57Bl/6 spleen cells, but not by bone marrow cells. Absorption with C3H thymus and L1210 cells was not attempted, and, therefore, the extent of the response to H-2^b antigens on EL4 cells cannot be estimated. A striking fact is the total absence of measurable C'ICC against C3H thymus cells despite the strong humoral response against them.

The results shown indicate that (a) AKR mice immunized with EL4 produce humoral

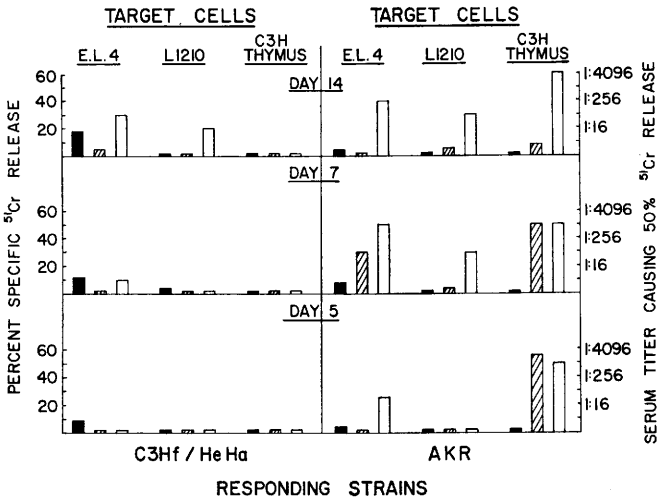

FIG. 2. Comparison of the immune response of female C3Hf/HeHa and AKR mice to the C57BL/6 lymphoma EL4. *In vitro* cytotoxic tests were performed using EL4, L1210, and C3Hf/HeHa thymus cells as targets. Responding mice were given a single ip inoculation of 3 $\times$ 10^7 EL4 cells on day 0. The mice were sacrificed on the days indicated. C'ICC (5) was assayed by incubating spleen cells and labeled target cells at a 100:1 ratio for 4 hr (solid bars). C'DCC (6) activity was assayed by incubating spleen cells and target cells at 100:1 ratio for 1 hr followed by the addition of rabbit complement and further incubation for 1 hr (hatched bars). SA (6) was assayed by incubating target cells and the appropriate serum dilutions for 1 hr followed by the addition of rabbit complement and further incubation for 1 hr (empty bars). C'ICC and C'DCC values are expressed as percent specific release (left ordinate). SA is expressed as the highest serum titer causing 50% specific release (right ordinate). Five mice were in each group for each experiment, and the corresponding spleens were pooled for the assays. Each experiment was repeated a minimum of three times; thus, each bar represents the average values from a minimum of 15 mice.

responses directed against "C" antigens and mostly against the θC3H antigen that can be measured separately from "background" responses directed against H-2^b "private" antigens. However, no C'ICC against "C" and θC3H antigens could be measured with L1210 and C3H thymus cells. (b) C3H mice immunized with EL4 develop a strong and rapidly appearing C'ICC mainly directed against the H-2^b "private" antigens. The "C" antigen elicits only a humoral response in these mice and no C'ICC (at least when tested on L1210 cells). The humoral response presumably directed against "C" in C3H mice does not seem to impair the C'ICC response directed against the H-2^b "private" antigens. (c) DBA/2J mice immunized with EL4 raise a strong, rapidly appearing humoral response directed against the H-2^b "private" antigens but almost no C'ICC (at least during the first 12 days).

Discussion. In this study, an attempt has been made to analyze the role of the various antigenic specificities present on a nucleated cell in eliciting *in vivo* cellular and humoral responses, which were then measured *in vitro*. EL4 leukemia cells were used as immunogens, and three specificities were considered, viz. H-2^b ("private"), θC3H and the "public" antigens of H-2 coded by the D region called here antigen "C", common to C57BL/6, and DBA/2 cells. In order to obtain immune responses directed toward three, two, or one of these antigens, AKR, C3Hf/HeHa, and DBA/2J mice were used, respectively. In the *in vitro* tests, three target cells were used as indicators as follows: EL4 for H-2, L1210 for "C", and C3Hf/HeHa thymocytes for θC3H antigen. The *in vitro* assays were based on the ^{51}Cr release from prelabeled target cells and measured the complement-independent cellular cytotoxicity in the presence of

immune spleen cells (5) and the cytotoxic humoral response in the presence of either immune cells or immune serum and heterologous complement (6). While the C'ICC is generally considered to be the expression of a T-cell response (5), the C'DCC measures cytotoxic antibody responses. Although the C'DCC could not be reduced by treatment of effector cells with anti-θ antiserum (6), no definitive evidence is available as to the cell types involved in generating the responses measured by this test.

All the antigens examined elicited humoral antibodies during a primary response which could be detected with any of the target cells expressing the specificities considered. On the other hand, the C'ICC elicited by EL4 could be demonstrated only with EL4 cells, and not with cells expressing only part of the H-2 specificities [the "C" antigens shared by EL4 (H-2^b) and L1210 (H-2^d)] or the alloantigen θC3H. The fact that no C'ICC against antigen "C" was demonstrated with L1210 may reflect low sensitivity of the test, low density of antigen on target cells, and/or relative resistance of this target cell to immunolysis. On the other hand, C3H thymus cells are known to have a high θC3H density on their surface (11). Therefore, the absence of an AKR C'ICC directed against the θC3H antigen when C3Hf/HeHa thymus cells were used as target cannot be explained by a low density of the θ antigen on these cells. Neither can it be explained by a low density of antigen on the immunizing EL4 cells, because of the high anti-θ humoral response elicited in the same host. Even when C3Hf/HeHa thymus cells were used to immunize AKR mice, no C'ICC was detected (6). Thus, regardless of whether the response was elicited across or within H-2, no anti-θ C'ICC could be detected; this also indicates that H-2 does not play a "helper" function (12) in relation to a complement-independent cellular response to θ. It is of interest in this respect that skin grafts between strains of mice differing only by the θ alloantigens are not rejected although cytotoxic anti-θ antibodies are produced by the recipient (13). It is conceivable that the capacity of some antigens to elicit *in vivo* transplantation immunity might be identified by their capacity to stimulate a C'ICC detectable *in vitro*. In this study, both transplantation and nontransplantation antigens could be detected by humoral antibodies, while only the transplantation antigens could be detected by the C'ICC. Although the possibility that multiple immunization with nontransplantation antigens also elicit a C'ICC response was not tested, it is of interest that the rapidity and magnitude of the humoral response correlated with the capacity of the animals to reject rapidly growing leukemia allografts, at least in the cases of L1210 (6) and EL4 in the strains of mice tested.

In conclusion, this investigation indicates that the *in vitro* tests used provide a means of detecting not only the host responses to a polyantigenic cellular system as a whole, but also those to each individual set of antigenic components. Whether or not the approach tested in this investigation might lead to development of *in vitro* correlates of transplantation immunity requires further study.

Summary. The immune responses elicited during a primary immunization in DBA/2J, C3Hf/HeHa, and AKR mice by allografts of the C57BL/6 lymphoma EL4 were studied *in vitro*. The ^{51}Cr release assays were used to measure the complement-independent cellular cytotoxicity (C'ICC), the complement-dependent cellular cytotoxicity (C'DCC), and the serum cytotoxicity (SA). The following observations were made: (a) C'ICC was detected against the major histocompatibility antigens (H-2) in all three strains; (b) no C'ICC was detected against the θC3H alloantigen in AKR mice or against the D-region "public" antigens of H-2 (called here "C") in C3H or AKR mice; (c) C'DCC and/or SA were detected against each of the three antigens in the appropriate responder strains; (d) no major interference was observed between the concomitant complement-dependent humoral response to a nontransplantation alloantigen (θC3H) and H-2, and the cell-mediated response (C'ICC) to H-2; (e) the order of appearance of the C'ICC and C'DCC were different in the three responder strains.

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