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SPECIFICITY OF CYTOTOXIC HETEROLOGOUS ANTISERUM TO CULTURED HUMAN CANCER CELLS

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Both negative (1–5) and positive (6–9) evidence may be found in the literature for the occurrence of tumor-specific cytotoxic antibody in heterologous antisera to mammalian tumor cells. The negative evidence appears to have been due to the intervention of species-specific antibody. In a further examination of this question, we have studied the cytotoxicity and absorption reactions of rabbit antisera to cultured human cell lines and strains. Our experiments centered around the use of cell cultures of three types of tumors: sarcoma (SV40 virus-transformed human embryonic lung), carcinoma (HeLa), and lymphoma (EB2).

MATERIALS AND METHODS

Cell lines and strains. VA4 (SV40 virus-transformed cell line) was supplied by A. J. Girardi; HeLa, by W. H. Murphy; EB2, by A. J. Girardi and P. H. Moorhead; EB3, by P. H. Moorhead; WI-26 (human embryonic lung), by L. Hayflick. J-111, KB and Henle intestine cell lines were obtained from Baltimore Biological Laboratories; HEp-2, from Microbiological Associates. LuCa-1, which originated from a lung carcinoma, was grown by us from an operating room specimen obtained at this hospital through the collaboration of Dr. Clark E. Brown, chief pathologist, and staff surgeons, as were LymSa-1, LymSa-2 and LymSa-3 (lymphomas), HEL-1 (embryonic lung), and HESpl-1 (embryonic spleen). The cells were cultured by standard procedures in BME Earle's-10% calf serum in 1-L Blake bottles, or for the lymphomas, in suspension in RPMI-1629-10% calf serum in Erlenmeyer flasks, and stored at −87°C as described elsewhere (10).

Antisera. New Zealand male rabbits, 2 to 4 months of age, were inoculated intraperitoneally on 6 alternate days with suspensions of cultured cells, following the schedule used by Levi *et al.* (11). Whole cells, rather than homogenates or cell fractions, were used as antigen, based on the assumption (7) that antigens which might be unique to intact cells would thus best be retained. Preimmune sera were collected before the inoculations were initiated and immune sera were collected 1 week or later following the last inoculation. Adjuvant was not used. For certain studies, regular booster inoculations were given at 1- or 2-week intervals for the purpose of maintaining the antibody level.

For all lines and strains except the EB2, each inoculum consisted of the cells recovered from stored vials containing 3×10^7 cells; for the EB2 cells, because of their smaller size, twice as many cells were used. The contents of thawed vials were first diluted with 8 ml Hanks' BSS and centrifuged 10 min at 800 rpm at 4°C in a clinical type horizontal centrifuge. To remove most of the residual preservative which was present as a contaminant, the pellets of cells were resuspended in 10 ml fresh Hanks' BSS, centrifuged as before, and the washing process repeated a second time. The final pellet was suspended in 1 ml Hanks' BSS and injected in this form.

The first antisera which were studied were stored at −87°C. However, these preparations gradually lost activity. Subsequent antisera were lyophilized and stored at 4°C, under which conditions they were much more stable.

Cytotoxicity assay. Since the present study was carried out concomitantly with the development of the cytotoxicity assay (10), both a "conventional" procedure and a "modified" procedure were used. Tests for cross-reactions of antisera with cells other than those against which the antisera were originally prepared were always controlled by assays carried out simultaneously with the use of the homologous cells.

Absorption tests. In a typical absorption experiment, 1 ml suspension of washed cells, 1 ml antiserum, and 0.5 ml normal rabbit serum used as complement were mixed, incubated 60 min at 37°C, and centrifuged. The numbers of cells used are indicated below under Results. The complement was used undiluted. The medium used was Hanks' BSS. The control test of unabsorbed anti-

TABLE I

Comparison of titers of preimmune and immune sera[a]

Antiserum	Rabbit No.	Titers of Sera	
		Preimmune	Immune
VA4	1	2	12
	2	3	2
	3	3	92
HeLa	1	4	19
	2	13	22
	3	14	43
EB2	1	5	183
	2	3	130
	3	4	208

[a] Determined by conventional assay procedure (10).

serum consisted of 1 ml medium, 1 ml antiserum, and 0.5 ml complement, incubated and centrifuged the same as the absorbed antiserum. Finally, suitable aliquots of absorbed and unabsorbed antisera were assayed for cytotoxicity against target cells.

RESULTS

Comparison of titers of cancer cell preimmune and immune sera. Three sets of three rabbits each were inoculated with VA4, HeLa, and EB2 cells, respectively. Titers of preimmune and immune sera for the homologous cells are shown in Table I. The data reveal increases in titer as a result of the immunization and higher titers for the EB2 antisera than for the VA4 or HeLa antisera.

Tests for cross-reactions of cancer cell antisera. The results of tests for cross-reactions carried out with certain of the antisera referred to in Table I are shown by data for Experiment 1 in Table II. The values of the titers for the homologous target cells are consistently higher than those for the heterologous cells, thus providing evidence for specificity of the antisera for the lines of cells against which they were prepared. Immunization of a second set of rabbits yielded the results shown for Experiment 2 in Table II. The data, including those for the additional tumor cell line, LuCa-1, supported those of Experiment 1. The results obtained with cultured normal cells (HEL-1 and HESpl-1) demonstrated specificity also, although the comparatively low titers with the homologous cells suggested low immunogenicities of the antigens involved. The low immunogenicity of the normal cells was confirmed by the results of experiments with additional rabbits.

To explore further the question of specificity of the cytotoxic sera, additional lines and strains were used as sources of target cells. Among these was included the WI-26 strain which represented

TABLE II

Cross-reactions of cancer cell antisera

Expt. No.	Antiserum	Assay Method	Titers against Different Lines or Strains of Cells													
			VA4	HeLa	EB2	LuCa-1	HEL-1	HESpl-1	WI-26	KB	HEp-2	HeIn	J-111	EB3	Lym Sa-1	Lym Sa-2	Lym Sa-3
1	VA4	Conv.	*11*[a]	4	4	4	3	4									
	HeLa	Conv.	3	*12*	3	2	1	2									
	EB2	Conv.	15	12	*180*	10	14	10									
2	VA4	Conv.	*25*	3	2	2	2	4									
	HeLa	Conv.	2	*74*	3	3	1	2									
	EB2	Conv.	3	2	*264*	2	2	2									
	LuCa-1	Conv.	2	2	2	*31*	2	2									
	HEL-1	Conv.	2	2	2	2	*10*	2									
	HESpl-1	Conv.	1	2	1	2	2	*6*									
3	VA4	Mod.	*100*[b]	7	4				4	4	6	3	10	16			
	HeLa	Mod.	23	*100*[b]	14				6	16	2	5	*113*	5			
	EB2	Mod.	2	6	100[b]				1	2	2	1	4	*46*	10	0	*240*

[a] Values are italicized for titers obtained with homologous and strongly cross-reacting systems.

[b] Values of titers obtained in experiment 3 were "normalized" to 100 for the homologous systems and proportionate values were assigned for the heterologous systems.

TABLE III

Homologous and heterologous cell absorption of cytotoxicity toward homologous cells

Antiserum	Absorbing Cells	% Absorption with Different No. of Cells	
		3.0×10^6	0.3×10^6
VA4	VA4	92	56
	W126	73	37
	HeLa	66	14
	EB2	66	0
HeLa	HeLa	96	68
	W126	84	33
	VA4	76	10
	EB2	66	6
EB2	EB2	88 (81)[a]	60 (65)[a]
	W126	72 (68)	61 (64)
	HeLa	70 (52)	59 (65)
	VA4	47 (66)	64 (70)

[a] Values shown in parentheses were obtained in separate tests.

the precursor of the virus-transformed VA4 cell line. For this work, a third set of rabbits was immunized and booster inoculations were given. Since all cross-reactions for a given antiserum could not conveniently be carried out on the same day and under identical conditions, day to day variation in titer was encountered as was to be expected from general experience (10). The titers could, however, be evaluated by first being normalized as to give values of 100 for those obtained with the homologous systems and assigning proportionate values for those obtained in the cross-reactions. The normalized data for Experiment 3, shown in Table II, again supported the previously obtained evidence for specificity of antisera for homologous cells but with two exceptions, namely, the strong cross-reactions of HeLa antiserum with J-111 cells and of EB2 antiserum with EB3 and LymSa-3 cells.

The actual values observed in Experiment 3 for titers of VA4 antiserum with VA4 cells, HeLa antiserum with HeLa cells, and EB2 antiserum with EB2 cells, were around 1400, 4000, and 100,000 respectively. The use of an improved method of storage of antisera (lyophilization), improved method of immunization (booster inoculations), and improved assay method (modified method carried out in Hanks' BSS diluent) may each have contributed to the relatively high values of these titers.

Cross-absorptions of cancer cell antisera. Results of absorption tests in which homologous and heterologous cells were used as absorbing agents and in which the per cent absorption was determined against the homologous cells are shown in Table III. It is clear that heterologous cells as well as homologous cells were able to absorb cytotoxic activity against the homologous cells, showing a *qualitative* lack of specificity of the cellular antigens. However, particularly with the VA4 and HeLa antisera, it is also clear that absorption was *quantitatively* greatest when homologous cells were used as the absorbing agent, and therefore a certain degree of specificity was actually being evidenced. Additional experiments, not shown, demonstrated that essentially complete absorption was possible with either homologous or heterologous cells, provided sufficient numbers of cells were used.

DISCUSSION

The observed cytotoxic specificities, when considered without regard to the findings of the absorption studies, provide strong evidence for the existence of tumor-specific antigens and for differences between the antigens of individual lines and strains of cells. Differences in histocompatibility antigens might be suspect were it not for the fact that human isoantigens cannot, apparently, be distinguished with the use of heterologous sera (12). Our observation (Table II, Experiment 3) that VA4 antiserum was relatively ineffective against WI-26 cells, which represented the normal strain of cells from which the VA4 line was originally derived, also serves as evidence that the antibodies prepared against the different tumor lines were not directed against histocompatibility antigens. The cross-absorptions, considered without regard to the cytotoxic specificities, on the other hand, might be interpreted to suggest the intervention of species antigens. The simultaneous occurrence of cytotoxic specificity and cross-absorption might be explained by the hypothesis that tumor-specific antigen represents modified species antigen and that tumor-specific and species antigens might be sufficiently similar to one another to lead to cross-reactions exhibited by absorption but not to cross-reactions exhibited by cytotoxicity. Thus, the "fit" between tumor-specific or species antigens on the one hand and the antibody against any individual cell line on the other may be sufficiently good to permit absorption in all cases,

but not good enough to lead to cytotoxic damage except for the homologous system consisting of a given cell and the specific antibody prepared against it. The fact that absorption of cytotoxic activity by homologous cells can be shown to be quantitatively greater than that by heterologous cells also gives support to the hypothesis that the fit between homologous antibody and antigen is better than that with heterologous combinations. There is precedent for the phenomenon of cytotoxic-negative-absorption-positive antibody as related to heterologous target cells in the report of agglutination-negative-absorption-positive antibody for leukocytes and of cytotoxic-negative-absorption-positive antibody for lymphocytes (13). Also, there may be an analogy between our findings and those of Paul *et al.* (14) who showed that anti-DNP-BSA antibodies, produced in rabbits made tolerant to BSA, possessed a higher avidity for DNP-BSA than for the cross-reacting BSA.

The low cytoxicity of tumor antisera for most of the cell lines other than those against which the antisera were prepared, and for all of the cell strains tested, suggests that as far as immunogenic stimulation of formation of cytotoxic antibody is concerned, the influence of tumor-specific antigen was dominant over that of species antigen. The relatively low titers of the normal cell antisera compared to those of tumor antisera also suggests a lower immunogenicity of species, compared to tumor-specific antigen. The explanation for the cytotoxicity cross-reactions between EB2 antiserum and EB3 and LymSa-3 cells in the one instance and between HeLa antiserum and J-111 cells in the other may, therefore, lie in similarities of tumor-specific antigens, rather than of species antigens. The implication of species antigens in the cytotoxicity cross-reactions cannot, however, be ruled out by the present data. Similarities between antigens of EB2, EB3, and LymSa-3 cell lines are reminiscent of similarities between antigens of mouse lymphosarcomas (15). Similarity between HeLa and J-111 antigens was unexpected.

No effort was made to prove the neoplastic nature of LuCa-1, LymSa-1, LymSa-2, or LymSa-3. Although more direct evidence is needed, it is suggestive that LuCa-1 showed an immunogenic activity comparable to that of HeLa and VA4 and greater than that of the normal strains, HEL-1 and HESpl-1, and that LymSa-3 showed cross-reactivity with EB2.

The apparent discrepancies in results obtained in different laboratories with respect to cytotoxic specificity and cross-absorption, using heterologous antisera, may well be attributable to the use of a variety of animals and immunization regimens for production of antisera. Thus guinea pigs, chickens, cows and rabbits have each been used by different investigators and the immunization regimens have varied from a single injection of antigen into chickens (6) in an attempt to avoid the tendency of multiple injections to reduce the specificity of antisera, to multiple injections of relatively large amounts of antigen into rabbits (3), which may have been responsible for the lack of tumor specificity for antisera obtained under such conditions. Discrepancies in results of absorption tests may sometimes have been due to inadequate attention to quantitative aspects of such tests. For example, as our own work has shown, where large enough numbers of cells are used complete absorption may be expected with heterologous as well as with homologous cells, while with fewer cells absorption by heterologous cells may be expected to be significantly less than that with homologous cells.

If it is assumed that the differing results reported in the literature are equally valid and conflict only because of the use of different experimental conditions such as those discussed above, it may be concluded that it is possible to obtain cytotoxic heterologous antibody having varying combinations of properties: a) tumor-specific cytotoxicity with little cross-absorption by heterologous cells of the same species (6–9), b) tumor-specific cytotoxicity with significant cross-absorption by heterologous cells of the same species (present work), or c) only species-specific cytotoxicity, with more or less complete cross-absorption by heterologous cells of the same species (1–5).

SUMMARY

Rabbit antisera prepared against representative cultures of human cancer cells (SV40 virus-transformed, HeLa, and EB2 cells) showed high cytotoxic specificity for homologous cells when tested against these and cells of other lines and strains. Similar results were observed with antisera against a new cell culture originating from human lung cancer. Antisera against cultures of normal human cells also showed specificity, but relatively low titers. Cross-reactions were observed between HeLa cell antiserum and J-111

cells and also between EB2 antiserum and EB3 cells and a new cell culture originating from human lymphosarcoma.

The cytotoxic activity of the antisera was absorbed by cells of human lines or strains other than those used to produce the antisera, but more effectively by homologous than heterologous cells. The apparent ambiguity arising from the simultaneous occurrence of high specificity of antisera with respect to cytotoxicity and low specificity with respect to absorption is discussed.

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