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Noncytolytic n-butanol Extraction of Soluble Murine Colon Tumor Rejection Antigens

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SUMMARY

Tumor rejection antigens (TRA) of murine colon tumors were extracted noncytolytically using n-butanol. When C-C26 and C-C36 were treated for 15 min with 2.5% n-butanol, soluble antigens were extracted from their membranes without cellular lysis, and the treated cells with this procedure showed the same proliferation as in the untreated cells. The immunogenicity of the extracted antigens was assessed in *in vivo* tumor transplantation assays. The data showed that the extracted antigens maintained their immunogenicity as tumor rejection antigens, and were more potent than the antigens extracted by a detergent Nonidet P40. In addition to the fact that n-butanol was easy to remove from the extracts, this extraction procedure was considered to be a new promising method to obtain the soluble tumor antigens effectively including TRA without cell lysis and the antigenicity.

Key words: Tumor rejection antigens, N-butanol extraction

INTRODUCTION

It is still difficult to obtain solubilized tumor rejection antigens (TRA) without loss of their immunogenicity *in vivo* and *in vitro*. During the past two decades, there were various reports that demonstrated effective extraction of soluble TRA from the cell surface membranes or cytosol fraction of the cells by various agents including hypertonic solution and detergents (2, 8, 9, 13, 14). The antigens extracted with these agents showed a relatively high immunogenicity *in vivo*. However, it also showed that the antigens extracted were often prone to aggregate in the solution, and were not amenable to the biochemical fractionation procedures (9). Furthermore, the antigens in certain occasions were denatured to a certain extent during the fractionation, and could no longer be employed in the experiments.

Moreover, when the detergents such as a nonionic Nonidet P40 (NP40) were used to solubilize the tumor antigens of the cells, it was usually very difficult to remove these detergents from the antigen solution, since they do not readily pass through a dialyzing membrane.

In the present report we describe a new effective extraction procedure using n-butanol of TRA from murine tumor cells without cellular lysis. The n-butanol was readily removed by dialysis from the extract solution (12). The antigens extracted by this method effectively maintained their immunogenicity, and were used for the bioassays *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Mice. Inbred BALB/c mice used in the experiment were obtained from CLEA Japan Co., Shizuoka, Japan, and some mice were maintained and raised in our laboratory. In the transplatation assay, 6 to 8 week-old male mice were employed.

Cells. Four tumor cells of BALB/c origin were used in the experiment. Meth A is a methylcholanthrene-induced fibrosarcoma, and can grow rapidly in ascitic form (6). Usually, the cells were aspirated from the mouse abdominal cavity, washed 3 times with PBS, and used for the assay. *In vitro* and *in vivo* growth characteristics as well as tumor rejection profiles of cultured murine colon tumors of BALB/c origin, C-C26 and C-C36, were previously reported (10, 11). Colon tumor C26 is a N-nitroso-N-methylurethance-induced tumor and by histopathology an undifferentiated carcinoma. Colon tumor C36 is a 1, 2 dimethylhydrazine dihydrochloride-induced tumor with the histopathology of a well differentiated adenocarcinoma. Cultured C-C26 and C-C36 were established from transplantable colon tumors C26 and C36, respectively. In addition, another colon tumor, C51, was adapted to grow *in vitro*. This is named C-C51 and is less immunogenic and not cross-reactive against C-C36 as well in the transplantation assays. These cell lines were kindly provided by Dr. Mark K. Wallack, Mount Sinai Medical Center, Miami Beach, Fl., and were grown in Eagle's modified medium with 5% fetal bovine serum and L-glutamine (292 μg/m*l*). Single cell suspensions of tumor cells were obtained by trypsinization (0.05% trypsin-0.02% EDTA) of confluent monolayers. The cells were washed two or three times with PBS and resuspended in appropriate volumes of PBS for the assays.

Assessment of antigen extraction, cell viability and proliferative capability by n-butanol treatment of cells. Approximately 10^7 cells/m*l* in PBS were treated with a final concentration of 1.0, 2.0, 4.0 and 8.0% n-butanol (Nakarai Chemical Ltd., Kyoto, Japan) for 5 min. We also treated the cells for 30 and 60 min with a final concentration of 2.0% n-butanol. The supernatants and these treated cells

were assessed *in vitro* for three parameters, namely protein content of the supernatants, cell viability and proliferative potentials of the cells. Immediately after the cells were treated with the each experimental procedure described above, the solutions were centrifuged for 5 min at 500G. The supernatants were then centrifuged for 20 min at 2000G, dialyzed 24 hr against 100 volume of PBS, and were partially concentrated against 50% sucrose in PBS. It was redialyzed 24 hr against PBS, and ultracentrifuged at 160,500G for 1 hr (12). The protein content of supernatants was measured by the method described by Lowry *et al.* (4). Treated cells were tested for their viability by trypan blue dye exclusion and the proliferation *in vitro* except Meth A tumor cells. 10^6 viable cells treated with various n-butanol concentrations in 5 m*l* medium supplemented with 5% fetal bovine serum were cultured for 72 hr in tissue culture flasks (Falcon #3024, Falcon Plastics, Oxnard, CA), and the number of proliferated cells at the bottom of the flasks were determined on a hemocytometer after trypsin treatment.

We examined in a separate experiment the efficiency of antigen extraction by 2.5% n-butanol. 10^7 cell/m*l* were treated for 5, 15, 30 and 60 min with a final 2.5% n-butanol concentration. Using the same manner of assessment described above, the protein content of the supernatants and the viability of treated cells were decided.

Antigen extraction by 2.5% n-butanol treatment of cells. Extraction of TRA by n-butanol from colon tumor cells was performed with 2.5% n-butanol treatment for a duration of 15 min for C-C26 and C-C36, and for less than 5 min for C-C51 cells, because treatment of cells with this concentration and duration provided a certain amount of protein yields without cellular lysis as shown in the results (Table 4). After concentration with 50% sucrose, the extract was dialyzed against PBS and finally ultracentrifuged. This was designated crude n-butanol extract (CBE) (3).

Solubilization of TRA by NP 40. TRA was also isolated from colon tumor cells by nonionic NP40 (Nakarai Chemical Ltd., Kyoto, Japan) as described by Natori *et al.* (6), except that the cell disruption by N_2 bomb was omitted.

Determination of the immunogenicity of CBE and NP40 extracts. To test the immunogenicity of TRA extracted from colon tumor cells by n-butanol and NP40, we immunized subcutanously 10 to 12 mice/group into their left backs twice at weekly intervals with 25-30 μg crude proteins of C-C26, C-C36 and C-C51 extracted by these agents. A week after the second immunization, mice were challenged subcutaneously into their right backs with 10^5 C-C36 cells. Following the challenge, the animals were examined at least once a week to record the onset of tumors (tumor incidence).

Data analysis. The P values for the tumor growth were calculated by Fisher's

exact provability test.

RESULST

Effect of n-butanol treatment on protein yield, viability and proliferation of cultured colon tumor lines. To examine the solubilization of antigen by n -butanol from the cells, we treated cells with various concentration of n-butanol in PBS as well as with various durations of treatment. The data shown in Table 1 indicate that treatment of C-C26 cells by 1.0 and 2.0% n-butanol for 5 min did not show any effect on the cells. However, 4.0% n-butanol treatment for 5 min

Table 1 *Effect of n-butanol treatment on protein yield, cell viability and proliferation of colon tumor C-C26.*

butanol concentration (%)	treatment time (min)	protein yield (μg/10^6 cells)	viability (%)	proliferation[a)] ($\times 10^6$)
0	5	4.2	95.0	6.1
1	5	4.3	96.5	7.3
2	5	4.3	92.5	6.4
4	5	11.1	7.2	0.03
8	5	16.6	0.	0.01
2	30	9.0	68.6	1.4
2	60	16.0	26.7	0.9

[a)] 10^6 viable cells treated with n-butanol in 5 m*l* medium supplemented with 5% fetal bovine serum were cultured for 72 hr in tissue culture flasks, and the number of proliferated cells at the bottom of the flasks was determined on a hemocytometer after trypsin treatment.

Table 2 *Effect of n-butanol treatment on protein yield, cell viability and proliferation of colon tumor C-C36.*

butanol concentration (%)	treatment time (min)	protein yield (μg/10^6 cells)	viability (%)	proliferation[a)] ($\times 10^6$)
0	5	4.6	95.5	5.5
1	5	4.3	94.1	6.8
2	5	4.0	92.5	6.0
4	5	7.0	48.8	0.03
8	5	9.7	0.	0.01
2	30	5.3	84.4	6.6
2	60	9.9	73.0	1.2

[a)] See footnote in table 1.

reduced the viability as well as proliferation of C-C26. Although the protein yield was increased, it appeared that the vast majority of protein was derived from the cytoplasma. Treatment of 2.0% n-butanol for 30 min resulted in an increased protein yield. However, this also showed a reduced cell viability (68.6%). Table 2 shows the results of n-butanol treatment of C-C36, indicating almost the same pattern of reactivity as that of C-C26. It is demonstrated in Table 3 that C-C51

Table 3 *Effect of n-butanol treatment on protein yield, cell viability and proliferation of colon tumor C-C51.*

butanol concentration (%)	treatment time (min)	protein yield (μg/10^6 cells)	viability (%)	proliferation[a)] ($\times 10^6$)
0	5	3.7	84.1	3.1
1	5	4.0	88.2	3.6
2	5	7.0	63.3	2.1
4	5	13.1	3.9	0.04
8	5	15.6	0.	0.01
2	30	41.0	15.5	0.05
2	60	66.0	2.0	0.03

[a)] See footnote in Table 1.

Table 4 *Effect of 2.5% n-butanol treatment on protein yield and coll viability of colon tumor lines.*

cells	treatrent time (min)	protein yield (μg/10^6 cells)	viability (%)
C-C26	0	3.6	95.7
	5	1.2	96.6
	15	6.5	92.5
	30	7.7	67.5
	60	10.9	57.6
C-C36	0	2.3	98.4
	5	1.5	97.8
	15	3.8	98.5
	30	4.1	93.8
	60	7.3	86.2
C-C51	0	1.7	91.3
	5	3.9	67.7
	15	12.4	24.1
	30	14.8	12.3
	60	20.0	0.

cells were highly vulnerable to n-butanol treatment. In fact, the protein yield was increased to a certain amount, while the viability and cell proliferation of the cells were moderately reduced with 2.0% n-butanol for 5 min. Collectively, the data shown in Table 1-3 indicates that (1) 1.0% n-butanol was not effective for antigen extraction from the cells, (2) 2.0% n-butanol may be effective, but there was no protein yield increase without cellular lysis, and (3) n-butanol more than 4.0% in PBS was toxic to the cells even in 5 min treatment.

These results suggest other trials for n-butanol treatment. Because it seemed that a critical concentration of n-butanol for the antigen extraction was between 2.0 to 4.0%, we examined the effectiveness of 2.5% n-butanol to these cells. Table 4 indicates that C-C26 and C-C36 antigens were effectively extracted without cellular lysis and loss of proliferative capability by 2.5% n-butanol treatment for 15 min. C-C36 still shows a high viability even in 30 min treatment, but a marked increase of protein yield was not detected. C-C51 was vulnerable to n-butanol treatment. Usually this line showed a slight amount of protein yield with 2.5% n-butanol for less than 5 min. These data indicated that the extraction of antigens by n-butanol from colon tumor cells should be done with 2.5% n-butanol treatment for a duration of 15 min for C-C26 and C-C36, and for a duration of less than 5 min for C-C51, respectively. After concentration with sucrose, the extract was dialyzed against PBS and finally ultracentrifuged.

Effect of 2.5% n-butanol treatment on protein yield and viability of ascitic Meth A cells. We showed the results of n-butanol action on the antigen extraction from murine colon tumor lines, C-C26, C-C36 and C-C51. These lines were all *in vitro* cultured cells. To assess whether n-butanol is also effective to *in vivo* grown cells, we examined the protein yield and viability of Meth A ascitic cells by 2.5% n-butanol treatment. Table 5 showed that the extraction officiency of Meth A antigens from the cell surface was in the same pattern of the reactivity as that of C-C26 and C-C36 *in vitro* cultured lines. This suggests that n-butanol extraction of membrane antigens could be applicable for *in vivo* as well as *in vitro* grown cells.

Immunogenicity of CBE of colon tumor cells. BALA/c mice were immunized subcutaneously with 25-30 μg of CBE of C-C36, C-C26 and C-C51. For

Table 5 *Effect of* 2.5% *n-butanol treatment on protein yield and cell viability of in vivo grown Meth A cells.*

	treatment time (min)				
	0	5	15	30	60
protein yield (μg/10^6 cells)	5.4	4.4	8.0	12.9	20.2
viability (%)	90.9	89.1	88.2	82.8	45.5

Table 6 *Challenged C-C36 tumor incidence of mice immunized with various extracts from murine colon tumors.*

immunized with 25-30 μg of extract protein	tumor incidence of C-C36 challenged at 3wks	4	5	6	7
	(No. of mice with tumor/total No. of mice)				
C-C26 CBE[a)d)]	0/10[c)]	8/10	8/10	8/10	8/10
C-C26 NP 40E[b)d)]	2/10[c)]	8/10	9/10	9/10	10/10
C-C36 CBE[d)]	1/10[c)]	3/10[c)]	3/10[c)]	4/10[c)]	4/10[c)]
C-C36 NP 40E[d)]	2/11[c)]	5/11[c)]	6/11	7/11	7/11
C-C51 CBE[d)]	9/10	10/10	10/10	10/10	10/10
C-C51 NP 40E[d)]	9/11	10/11	10/11	10/11	10/11
none (PBS)[d)]	9/12	11/12	11/12	11/12	11/12

[a)] Crude n-butanol extracts; [b)] Nonidet P40 extracts;

[c)] $P<0.05$

[d)] Mice were immunized with the extracts or PBS twice at weekly intervals, and were challenged one week after the second immunization with 10^5 C-C36 cells.

comparison of the relative potency of the immunogenic activity of tumor rejection antigen, NP40 extracts with the same protein concentration of these tumors were injected into another group of mice. These mice were challenged with 10^5 C-C36 cells a week after the second immunization. The data (Table 6) demonstrated that C-C36 CBE was highly immunogenic, and half of the mice immunized with C-C36 CBE rejected the challenged C-C36 tumors. In addition, C-C26 CBE was shown to be also immunogenic, indicating that the antigens were cross-reactive against C-C36 cells in terms of tumor rejection. This result may agree with that of our previous studies (11) in which C-C36 and C-C26 were demonstrated to have cross-reactive TRA in the cross-transplantation experiments. Although the extract antigens of these cells by NP40 were clearly immunogenic and cross-reactive between C-C36 and C-C26 extracts, it appeared that their relative potentials with respect to the immunogenic activity were lower than those of CBE, since mice immunized with C-C36 extracts by NP40 exhibited reduced tumor incidence with statistical significance at only the third and fourth week after the challenge. The results of tumor incidence of mice immunized with C-C51 CBE or NP40 extracts suggested that the presence of cross-TRA between C-C36 and C-C51 cells were not found.

DISCUSSION

The purification and characterization of the molecular nature of tumor specific rejection antigens (TRA) is crucially important for the immunological manipula-

tion of the host immune responses against tumors and to understand the background of these antigens as the gene products on the process of malignant transformation of cells (7). It has been reported in animal tumor models that chemically induced tumors have individually specific TRA, and that these antigens were solubilized by several agents and semipurified with the biochemical procedures (2, 8, 9, 13, 14).

However, it has been usually very difficult to solubilize TRA on the cell surface in high yields and to maintain their immunogenicity. Even though tumor antigens were successfully extracted in a solubilized form by a certain agent, it was found that the antigen molecules were inclined to aggregate among the extracted molecules (9). In these cases, the purification of TRA molecules could no longer be anticipated in spite of many attempts to remove the aggregation of antigens (5). Furthermore, the solubilizing agents including nonionic detergents that were used frequently for analyzing TRA are highly toxic to the cells. We should also know that these detergents in the extract solutions are less dialysable, hence we must be very careful in using the antigens extracted by these agents in *in vivo* and *in vitro* assays. Moreover, it suggests that the vast majority of extracted protein by these agents are derived from intracytoplasmic cell matrix (cytosol) rather than cell surface membranes (1). Therefore, there is a possibility that the certain enzymes that have protease-like activity in the extracted solutions may destruct or denature the immunogenic molecules from the cell surface membranes. The tumor antigens were also extracted by treatment with hypertonic solubilizing agents such as 3 M KCl, and eventually these were readily removed from the solution by dialysis. However, it should be noted that the intracytoplasmic protein extracted by this agent might denature the intact immunogenic molecules of tumor antigens from the cell surface as well.

We demonstrated here that antigens on the cell surface membrane are effectively extracted by n-butanol under a certain condition without cellular lysis. Although the extraction officiency of antigens from the cell surface membranes may depend upon the cells, it could be applicable to *in vivo* grown as well as *in vitro* grown tumor cells. It was suggested that the antigens extracted with this procedure are obtained already in a semi-purified form, since there could be no contaminations of intracytoplasmic proteins in the extracted solution. Furthermore, this means that the antigens solubilized by this procedure could readily maintain their immunogenicity, and might be amenable to further biochemical procedures for the purification of antigens. Our study described in another paper (12) and others (3) agree with the above advantages of this method. It was also shown that the antigen molecules extracted by 2.5% n-butanol treatment of cells rarely aggregated by themselves when 5 mM EDTA was added to the solution. Moreover, n-butanol is readily removable from the extracted solution. This suggests that the n-butanol

extracted antigen solution is not toxic, and could be useful for *in vitro* as well as *in vivo* bioassays. The findings demontrated in this report may lead to promising trials for the active immunotherapy in cancer patients.

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