*Proc. Nat. Acad. Sci. USA*
Vol. 68, No. 6, pp. 1372–1375, June 1971

# Suggestive Evidence That the "Blocking Antibodies" of Tumor-Bearing Individuals May Be Antigen–Antibody Complexes

**(Moloney sarcoma/methylcholanthrene-induced/adsorption/elution)**

H. O. SJÖGREN, I. HELLSTRÖM, S. C. BANSAL, AND K. E. HELLSTRÖM

Division of Immunology, Fred Hutchinson Cancer Center, Pacific Northwest Research Foundation;
Departments of Microbiology and Pathology, University of Washington Medical School, Seattle, Wash. 98105

*Communicated by George D. Snell, April 2, 1971*

**ABSTRACT** **Sera from mice carrying progressively growing sarcomas induced by Moloney virus or methylcholanthrene can block the cytotoxic effect of lymphocytes immune to the tumor-specific antigens of the respective neoplasms. The blocking effect can be specifically removed by absorbing sera with the respective types of tumor cells, and it can be recovered from these cells by elution at low pH. If the low pH is maintained, it is possible to separate out a low and a high molecular weight fraction from the eluates. If the fractions are added to the target cells for 45 minutes and then removed, neither of these fractions can block lymphocyte-mediated cytotoxicity, while a 1:1 mixture of them has a specific blocking effect. If they are admixed with the lymphocytes, incubated for 1 hr, and then allowed to incubate with the target cells and lymphocytes during the entire 2 days of the test, the low molecular weight fraction, as well as the mixture, but not the high molecular weight fraction, has a blocking activity.**

**It is suggested that the blocking factor in sera from tumor-bearing animals, as regularly tested, is an antigen–antibody complex, capable of binding to the target cells and/or reacting with lymphocytes immune to their antigens, thus blocking the lymphocytes' reactivity; the latter reaction is postulated to be of a temporary nature.**

Lymph-node cells and circulating blood lymphocytes from tumor-bearing animals and human patients have a specific cytotoxic effect on cultivated tumor cells from the same individuals (1–5). The cytotoxicity is almost the same as with lymphocytes obtained after the tumors have been removed.

Progressive tumor growth *in vivo*, despite a strong cell-mediated anti-tumor immunity demonstrable *in vitro*, has been attributed to a specific ability of the serum of tumor-bearing individuals to prevent target cell destruction by immune lymphocytes (2, 6–12); the mechanisms of the protection may be similar to those of the *in vivo* phenomenon of immunological enhancement (13). Sera from tumor-bearing animals, but not from tumor-free ones, can also abrogate the migration inhibition seen when peritoneal cell suspensions from specifically immune donors are exposed to tumor-antigen extracts *in vitro* (14).

The specificity of the blocking-serum activity suggests that it might be antibody-mediated. This conclusion is also supported by the findings that the blocking activity can be specifically removed by absorption of sera with tumor cells of the respective types (2), that the blocking fraction of the serum has the gel filtration characteristics of 7S immunoglobulin (2, 15), and that the blocking activity can be removed by incubation with antiserum to immunoglobulin (2).

One may hypothesize that blocking antibodies bind to the target tumor cells and thereby mask their antigens from detection by immune lymphocytes. Alternatively, one may visualize the blocking factors as antigen–antibody complexes, capable of acting on the target cells, the lymphocytes, or both, any action on the lymphocytes having to be of a temporary nature to account for the finding that lymphocytes from tumor-bearing individuals are specifically reactive *in vitro*. The second hypothesis is more compatible than the first one with the lack of detectable blocking activity in sera from mice whose (Moloney) virus-induced sarcomas have regressed (2–16), from rabbits whose (Shope) papillomas have regressed (17), and from mice and rats whose methylcholanthrene-induced and (polyoma) virus-induced sarcomas, respectively, have been removed (18), although such sera do contain antibodies that can bind to the respective type of tumor (16, 18)

This paper describes experiments with blocking sera from animals bearing progressively growing primary sarcomas induced by Moloney virus or transplanted methylcholanthrene-induced sarcoma (MCA-1050) isografts. The basic plan of the experiments was to try breaking up hypothetical antigen–antibody complexes by subjecting blocking sera to low pH, and subsequently separating their two components by ultrafiltration. The filters were chosen so as to be penetrable only by a hypothetical smaller "antigen" component and not by the larger "antibody" component; the calculations were based on the fact that the blocking activity of sera can be recovered in the 7S immunoglobulin fraction (2, 15). The low and the high molecular weight components, as well as a 1:1 mixture of the two, were then tested for blocking activity, by a microcytotoxicity assay (11).

## MATERIALS AND METHODS

Pools of sera were mixed with tumor cells obtained by trypsinization of monolayer cell cultures. After incubation with stirring for 1 hr at room temperature, the cells were spun down, separated from the supernatant (=absorbed serum), and washed 3 times with 15 ml of phosphate-buffered saline (0.01 M $NaPO_4$–0.15 M NaCl). The sedimented cells were subsequently resuspended in 20 ml of 0.08 M NaCl glycine buffer (pH 3.1) and incubated with stirring for 1 hr at room temperature. Cells were spun down and discarded, and the supernatant (=eluate) was treated in either of two different ways: (a) brought to pH 7.4 by the addition of 0.9 M

TABLE 1. *Blocking tests on ultrafiltrates of adsorbed and eluted Moloney sarcoma progressor and regressor serum*

| Serum preparation tested for blocking activity(a) | Preparations of three *progressor* serum pools(b) tested against four different target tumor cells(c) | | | | | | | | | | Preparations of regressor serum pool(b) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Serum pool I | | Serum pool II | | | | Serum pool III | | | | | |
| | Target tumor no. 1056 | | Target tumor no. 884 | | Target tumor no. 1066 | | Target tumor no. 1085 | | | | Target tumor no. 1061 | |
| | Standard expt.(d) | | Standard expt. | | Standard expt. | | Standard expt. | | Preincubation | | Standard expt. | |
| | % CI(f) | % abrog(f) | % CI | % abrog | % CI | % abrog | % CI | % abrog | % CI | % abrog | % CI | % abrog |
| Normal serum | 34.3*** | | 43.8*** | | 56.6*** | | 28.3*** | | 33.8*** | | 37.1*** | |
| Original MSV serum | 15.1 | 56.0** | 16.3 | 62.8*** | 26.3* | 53.5* | ... | ... | 3.1 | 90.8* | 42.7*** | 0(−15.1) |
| MSV serum absorbed with MSV tumor(c) | 35.0 | 0(−2.0) | 38.8*** | 11.3 | 64.6*** | 0(−14.1) | 33.0*** | 0(−16.6) | 34.2*** | 0(−1.1) | 50.2*** | 0(−35.3 |
| Eluate from MSV tumor cells(c) | 10.9 | 68.2*** | | | | | | | | | | |
| Eluate from MSV tumor cells (E100) | | | 32.8** | 25.1 | 50.8*** | 10.2 | 39.6*** | 0(−39.9) | 29.2*** | | 36.5*** | 1.6 |
| Eluate from MSV tumor cells (E10) | | | 44.8*** | 0(−2.2) | 70.4*** | 0(−24.4) | 25.5** | 9.9 | 1.5 | 95.6** | 42.1*** | 0(−13.5) |
| Eluate from MSV tumor cells (E100 + E10) | | | 12.7 | 71.0** | 31.3** | 44.7* | 7.7 | 72.8** | 16.0 | 52.7*** | 39.0*** | 0(−5.1) |
| Eluate E10 of MSV + eluate E100 of MCA tumor no. 1050(e) | | | | | | | 31.6*** | 0(−11.7) | | | | |
| Eluate E100 of MSV + eluate E10 of MCA tumor no. 1050(e) | | | | | | | 31.4*** | 0(−11.0) | | | | |
| Eluate of MCA tumor no. 1050(e) | | | | | | | 24.7*** | 12.7 | | | | |

(a) All sera were tested at a dilution of 1:6. Control serum was added to all eluates to a final concentration of 1:6. The dilution of the eluates was also 1:6 relative to the original serum.

(b) Progressor serum pools were obtained from BALB/c mice bearing a progressively growing Moloney virus-induced (MSV) sarcoma, while the regressor serum pool was derived from BALB/c mice whose MSV sarcomas had regressed spontaneously after a temporary growth period.

(c) Target cells were explants of MSV-induced sarcomas nos. 1056, 884, 1066, 1085, and 1061 derived from A/Sn mice and maintained by serial passage *in vitro*. A mixture of trypsinized cells from these tumors was used for adsorption–elution purposes in 10–25 × $10^6$ cells/ml serum.

(d) "Standard experiment" means that the target cells were exposed to the serum preparations, which were then removed before lymphocytes were added. "Preincubation experiment" means that serum preparation lymphocytes were mixed and incubated 1 hr at room temperature before the mixtures were added to the target cells.

(e) A serum from mice bearing a methylcholanthrene-induced (MCA) sarcoma no. 1050 was absorbed with no. 1050 tumor cells and eluate fractions were prepared in the same way as those from the MSV tumor cells.

(f) "% CI" indicates the percentage reduction of cell numbers with specifically immune as compared to control lymphocytes; the probability that the reduction seen with immune lymphocytes is due to chance is indicated. "% abrog" indicates the blocking effect of a given fraction (or serum); the probability that this blocking (as calculated from comparison with the lymphocyte effect seen with normal serum) is due to chance is indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 2. *Blocking tests on ultrafiltrates of adsorbed–eluted methylcholanthrene tumor MCA-1050 progressor serum*

| Material tested for blocking activity | | | Target tumor MCA-1050* | | | |
|---|---|---|---|---|---|---|
| | | | Expt. 1 | | Expt. 2 | |
| Serum preparation | | Dilution relative to original serum | % CI | % abrog | % CI | % abrog |
| Normal serum | | 1:6 | 40.7*** | | 47.2*** | |
| | | 1:12 | | | 50.3*** | |
| Original MCA-1050 progressor serum | | 1:6 | 21.1 | 48.2** | 9.7 | 79.4** |
| MCA-1050 progressor serum absorbed with MCA-1050 cells | | 1:6 | 55.9*** | 0 (−37.3) | 55.3*** | 0 (−17.2) |
| | | 1:12 | | | 39.1*** | 22.2 |
| MCA-1050 progressor serum absorbed with MSV sarcoma cells | | 1:6 | 26.4** | 35.1* | | |
| Eluate from MCA-1050 cells with adsorbed MCA-1050 progressor serum | E100 | 1:6 | 42.3*** | 0 (−3.9) | 48.5*** | 0 (−2.7) |
| | | 1:12 | | | 53.9*** | 0 (−7.1) |
| | E10 | 1:6 | 38.2*** | 6.1 | 47.3*** | 0 (−0.2) |
| | | 1:12 | | | 37.6** | 25.2 |
| | E100 + E10 | 1:6 | 5.4 | 86.7*** | −5.2 | 100*** |
| | | 1:12 | | | 1.0 | 98.0*** |
| Eluate from MSV sarcoma cells with adsorbed MCA-1050 progressor serum | E100 | 1:6 | 46.0*** | 0 (−13.0) | | |
| | E10 | 1:6 | 50.5*** | 0 (−24.1) | | |
| | E100 + E10 | 1:6 | 35.1*** | 13.8 | | |
| Eluate from MCA-1050 cells with adsorbed MSV sarcoma progressor serum | E100 | 1:6 | | | 45.8*** | 3.0 |
| | E10 | 1:6 | | | 46.3*** | 1.9 |
| | E100 + E10 | 1:6 | | | 46.2*** | 2.1 |

* The experiments were done the "standard way", and probabilities that differences obtained were due to chance were calculated, as described in footnotes to Table 1. By testing the same preparations in both the Moloney sarcoma and the MCA tumor systems, nonspecific toxic or stimulatory effects could be excluded as explanations of the findings obtained.

$NaHCO_3$, incubated at room temperature for 1 hr, dialyzed with 150 volumes of $NaPO_4$–NaCl, concentrated to the original serum volume, and sterilized by filtration; or (*b*) ultrafiltered at pH 3.1 through an Amicon (Amicon Corp., Lexington, Mass.) filter XM-100 (retaining molecules above about 100,000 daltons). The filtrate was saved for further processing and the retentate was washed with 20 volumes of glycine buffer on the XM-100 membrane to remove low molecular weight material. It was concentrated to about 10 ml, adjusted to pH 7.4 with 0.9 M $NaHCO_3$, dialyzed with 150 volumes of $NaPO_4$–NaCl in the filter chamber, concentrated to the original serum volume, and sterilized by filtration (=preparation E 100). This fraction should contain eluted molecules with molecular weights above 100,000, thus including immunoglobulin molecules. The saved filtrate of the initial filtration on XM-100 was adjusted to pH 7.4 by the addition of 0.9 M $NaHCO_3$, dialyzed on an Amicon filter PM-10 (retaining molecules above 10,000 daltons) with 150 volumes of $NaPO_4$–NaCl, concentrated to the original serum volume, and sterilized by filtration (=preparation E 10). This fraction should contain eluted molecules with molecular weights between 10,000 and 100,000, thus excluding immunoglobulin molecules. Equal parts of preparations E 10 and E 100 of each serum were mixed and incubated for 1 hr at room temperature in order to obtain reconstitution of possible antigen–antibody complexes.

The original sera and the various preparations obtained from them were tested for their capacity to block the cytotoxic effect of lymphocytes immune to the specific antigens of the tumors under study. In these tests our standard assay was used, according to which material to be tested for blocking activity was incubated with the target cells for 45 min, then poured off, and the target cells were exposed to test lymphocytes (11). In one series of experiments the different fractions were, instead, added to the lymphocytes and incubated with these for 60 min at 37°C before the lymphocytes (with the fractions still present) were tested for target-cell cytotoxicity.

Serum dilutions of 1:6 or 1:12 were used in the cytotoxicity assays. Control serum, obtained from untreated animals syngeneic with the donors of the test serum, was added to the eluates and their fractions to the same final concentrations as the original serum, which was assayed simultaneously (so that the "feeder" effect of serum should be similar). Relative to the original amount of the serum used for absorption and elution, all preparations were diluted 1:6 or 1:12, to facilitate comparison with the serum data.

## RESULTS AND DISCUSSION

Table 1 summarizes the results obtained with sera from animals bearing progressively growing Moloney sarcomas (progressors) and from animals whose Moloney sarcomas had regressed (regressors). As was reported (2), the progressor sera were found to block the immune lymphocyte effect. The blocking activity was absorbed out by 25–80 $\times$ $10^6$ Moloney sarcoma cells, and it was recovered in the unfractionated eluate of the tumor cells. While neither the E 100 fraction nor the E 10 had any significant blocking activity, the 1:1 mixture of the two fractions blocked. The same preparations of the regressor serum did not block.

A serum pool from mice carrying progressively growing MCA-1050 tumors was absorbed with and eluted from two types of tumor cells, the corresponding MCA-1050 cells and syngeneic Moloney sarcoma cells. The results (Table 2) show that all blocking activity was removed by absorption with the MCA-1050 cells, while the Moloney sarcoma cells did not remove any significant amount of activity, indicating

that the absorption effect was specific. Neither the E 100 nor the E 10 fractions of the eluate from the MCA-1050 cells showed any blocking effect when tested separately by the standard assay. Again, the mixture of the two fractions blocked. All preparations of eluates from the Moloney sarcoma cells were inactive. Neither was any blocking seen with eluates from MCA-1050 cells that had been incubated with Moloney progressor serum (instead of serum from mice carrying a growing MCA-1050 sarcoma). Mixtures of E 10 and E 100 fractions of Moloney progressor serum with E 100 and E 10 fractions of MCA-1050 progressor serum, respectively, did not block in the Moloney sarcoma system (Table 1). This indicates that the specificity of the blocking is associated with both fractions, E 10 and E 100.

Finally, one series of experiments was done (included in Table 1) in which the various serum preparations were not removed after incubation with the target cells, but were first incubated with the lymphocytes for 1 hr and then allowed to remain in the wells for the entire incubation period (2 days), before the microtest plates were stained. In this case, the E 10 fraction blocked, as did also the mixture of E 10 and E 100 fractions.

The present results demonstrate that the blocking activity of sera from tumor-bearing mice can be specifically absorbed by the respective tumor cells and subsequently eluted from them, while this does not happen when unrelated tumor cells are used for absorption. This gives increased support to the previous conclusion that the blocking-serum activity is at least partially mediated by antibodies (12). The fact that tumor cells incubated with Moloney regressor serum did not release any detectable blocking activity upon elution tends to exclude the possibility that the processing of the tumor cells used for absorption would in itself release any blocking activity. The experiments performed with sera and tumors from mice bearing either (Moloney) virus-induced or methylcholanthrene-induced sarcomas give even stronger support to this conclusion, since a blocking activity could be eluted only after the tumor cells had been treated with serum from donors bearing the same kind of neoplasms.

The finding that both the high and the low molecular weight fractions were inactive in the blocking tests, when assayed by our standard procedure wherein the fractions are removed after incubation with the target cells, while the reactivity was recovered when the fractions were mixed, is compatible with the hypothesis that the blocking effect is mediated by an antigen–antibody complex. In order to be detectable as blocking in our standard assay, such a complex would need to possess one free antigen-combining site, by which it can attach to the target cells, and its antigen part would have to be relatively small. It is conceivable that a hypothetical antigen–antibody complex could exert its effect by covering the target-cell antigens, but it seems more likely that the point of attack is the immune lymphocytes. The finding that the low molecular weight fraction (E 10) blocks by itself, if added to the test system and allowed to remain there during the whole incubation period, supports this view. It is conceivable that the blocking factor may also be an antigen–antibody complex in that case, the antibody part of which is synthesized by the lymphocytes. It is also possible, however, that the antigen alone may be sufficient for blocking if given the right opportunity to act on the lymphocytes. The accompanying report on studies performed in another system, involving lymphocytes from donors sensitized to H-2 antigens and tested with a slightly different technique, indicates that this may, indeed, be the case (19). If so, the antibody may serve the role of a "carrier" in the standard *in vitro* blocking experiments. Such a role may be of fundamental nature in the tumor system, as suggested by the fact that, in the present experiments, the blocking-serum effect can be completely absorbed out with the target cells.

Since we do not know whether the E 10 fraction represents an antigen and the E 100 fraction an antibody, and their mixture a complex, the present interpretation remains highly speculative. If it can be supported by further work, involving a detailed analysis of the properties of the fractions studied, a unifying concept of "enhancement" and allograft "tolerance" may evolve (12).

Finally, we want to point out that possibly analogous findings have been reported by Amos *et al.* (20), according to whom lymphocytes immune to H-2 antigens and incubated with target tumor cells that have been covered with specific anti-H-2 antibodies can become immunologically nonreactive. These authors have suggested that an immunosuppressive substance is formed by the lymphocytes or the target cells upon contact between antigen and enhancing antibody.

The skillful technical assistance of Mrs. Ingalill Mosonov, Miss Char Crossman, and Mrs. Mary Quentin is gratefully acknowledged.

This work was supported by grants CA 11742, CA 10188, and CA 10189 from the National Institutes of Health, by grant T 453 from the American Cancer Society, and by contract NIH 69-2061 from the National Institutes of Health to Dr. Charles McKhann, subcontracted to us.

1. Hellström, I., K. E. Hellström, and G. E. Pierce, *Int. J. Cancer*, **3**, 467 (1968).
2. Hellström, I., and K. E. Hellström, *Int. J. Cancer*, **4**, 587 (1969).
3. Hellström, I., K. E. Hellström, G. E. Pierce, and A. H. Bill, *Proc. Nat. Acad. Sci. USA*, **60**, 1231 (1968).
4. Hellström, I., K. E. Hellström, H. O. Sjögren, and G. A. Warner, *Int. J. Cancer*, **7**, 1 (1971).
5. Hellström, K. E., and I. Hellström, *Advan. Cancer Res.*, **12**, 167 (1969).
6. Hellström, I., K. E. Hellström, C. A. Evans, G. H. Heppner, G. E. Pierce, and J. P. .S Yang, *Proc. Nat. Acad. Sci. USA*, **62**, 362 (1969).
7. Heppner, G. H., *Int. J. Cancer*, **4**, 608 (1969).
8. Hellström, I., K. E. Hellström, and H. O. Sjögren, *Cell. Immunol.*, **1**, 18 (1970).
9. Sjögren, H. O., in "Immunity and Tolerance in Oncogenesis," *Fourth Quadrennial Perugia Cancer Conference*, ed. L. Severi (Perugia University Press, 1970), p. 551.
10. Sjögren, H. O., and K. Borum, *Cancer Res.*, in press (1971).
11. Hellström, I., H. O. Sjögren, G. A. Warner, and K. E. Hellström, *Int. J. Cancer*, **7**, 226 (1971).
12. Hellström, K. E., and I. Hellström, *Annu. Rev. Microbiol.*, **24**, 373 (1970).
13. Kaliss, N., *Int. Rev. Exp. Pathol.*, **8**, 241 (1969).
14. Halliday, W. J., *J. Immunol.*, **106**, 855 (1971).
15. Ankerst, J., *Cancer Res.*, in press (1971).
16. Hellström, I., and K. E. Hellström, *Int. J. Cancer*, **5**, 195 (1970).
17. Hellström, I., C. A. Evans, and K. E. Hellström, *Int. J. Cancer*, **4**, 601 (1969).
18. Sjögren, H. O., and S. C. Bansal, *Prog. Immunol.*, in press (1971).
19. Brawn, R. J., *Proc. Nat. Acad. Sci. USA*, **68**, in press (1971).
20. Amos, D. B., I. Cohen, and W. J. Klein, Jr., *Transplant. Proc.*, **2**, 68 (1970).