Effect of Immune Syngeneic Lymphoid Cells on Plating Efficiency of a Polyoma Virus-Induced Neoplasm (35070)

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Polyoma virus will induce tumors in some strains of newborn mice (1) and rats (2). Even resistant strains or adult animals become susceptible to polyoma oncogenesis after neonatal thymectomy (3, 4). The mechanism for this effect is thought to be the necessity of sufficient competent lymphoid cells to reject an arising tumor (5, 6). The tumor-enhancing effect of thymectomy can be overcome by passive transfer of immune or normal spleen cells (7) or thoracic duct lymphocytes (8). This restoration of resistance to tumor induction by lymphoid cells points to a significant role of cellular hypersensitivity in polyoma oncogenesis. In contrast with restoration with lymphoid cells, serum from immune mice has proved ineffective in protecting susceptible animals (7).

In an attempt to separate and observe the cellular and humoral factors in resistance to polyoma tumors the following study was undertaken. Previous workers have studied the effects of immune cells and serum on polyoma cells *in vitro* (9–11). In the present experiment, a given number of tumor cells were plated *in vitro* in the presence of sera and lymphoid cells. A reduction in tumor colonies was interpreted as an antitumor property of the added sera or cells.

Materials and Methods. Tumor. The virus-free fibrosarcoma designated BN SE3049 adapted to growth in tissue culture was obtained from Dr. Robert C. Ting, cloned (12), and maintained in Dulbecco's modified Eagle's media to which 10%, fetal calf serum (Hyland Laboratories) and 100 U penicillin/ml and 100 μg streptomycin/ml were added. The cloned cells were tested by classical rejection tests and contained polyoma virus-induced transplantation antigen. Cells were injected subcutaneously into syngeneic Brown Norway (BN) rats (Microbiological Associates) and this transplanted line used for tumor immunization.

Immunization. Lewis inbred strain rats 12 weeks old were given four 1 × 1 × 1-mm implants of BN SE3049 subcutaneously at 2-week intervals. Two weeks after the final injection, they were exsanguinated and the resultant serum filtered through a 0.22-μ Millipore filter and stored at —20° (Expt. IS, IIIS).

Twelve BN rats 16 weeks old under ether anesthesia were inoculated in the right hind footpad with a 1 × 1 × 1-mm fragment of the transplanted line of BN SE3049. Twelve weeks later the tumor was present in all 12 rats. Under ether anesthesia all right hind feet were amputated. This treatment in a preliminary study was observed to protect rats against subsequent tumor transplantation. Sixteen days later, four rats were sacrificed. Their sera, spleens, and draining lymph nodes were removed, pooled, and tested (Expt. IVS, IC). Six months later four more rats were killed and their lymph nodes and spleens tested (Expt. IIC).

BN rats used to carry transplant lines of BN SE3049 were bled. Their sera were tested (13) and no hemagglutination-inhibition titer against polyoma virus was detected. The pooled sera were filtered and stored at —20° (Expt. IIS, IIIS).

Age- and strain-matched, nontreated rats served as donors for normal cells and sera.

Plating efficiency assay. Two days before use, the BN SE3049 tissue culture line was

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subcultured with varying numbers of cells in several bottles. On the day of the assay, cells were recovered from that bottle in which 50% to 70% of the glass surface was covered by tumor cells. The monolayer was harvested at 37° with 0.05% trypsin in Earle's BSS (without Ca or Mg). After the trypsin was neutralized with fetal calf serum, a small fraction of these cells was suspended in Dulbecco's modified Eagle's medium to which 20% I.D.S. fetal calf serum (Hyland Laboratories), 100 U penicillin/ml and 100 μg streptomycin/ml has been added. The dilution of tumor cells was such that each plated milliliter of media contained 10 (trypan blue excluding) tumor cells (for plating 100 cells) or 16.7 cells (for plating 50 cells). Additional cells were added to compensate for the volume of added lymphoid cells or sera.

Cell suspensions of lymph node and spleen were made by pressing the tissue through a No. 60 wire mesh screen (14). The cells were rinsed in cold Earle's BSS (without Ca or Mg), resuspended in Earle's BSS, and viability-tested by trypan bblue dye exclusion. Nucleated, trypan blue-excluding cells were added to aliquots of the BN SE3049 cells such that a 200 to 1 lymphoid to tumor cell ratio was attained. This was usually done by diluting the lymphoid suspension so that the cells could be added to a volume of 0.10–0.50 ml.

Fresh or frozen sera were filtered at 0.22 μ through a Millipore filter and added to the tumor cell aliquot in a ratio of 1.0 ml serum to 100 tumor cells. If a serum pool proved active then it was heated to 56° for 30 min, stored at −20°, and filtered before use in the next experiment. Lyophilized rabbit complement (Lot #3025Cl, Hyland Laboratories) was reconstituted before use. Whenever complement was added, 0.10 ml of a 1 to 10 dilution was used.

After the reactants and tumor cells were mixed, aliquots were placed in tissue culture plates (Falcon Plastics). For some experiments 10 ml of media containing 100 tumor cells were placed in 60 × 5-mm plates; in others, 3 ml of media containing 50 tumor cells were placed in 35 × 10-mm plates. These were placed in a humidified incubator at 37° with 5% CO_2 and 95% air. After 7 days the plates were checked and any with bacterial or fungal contamination discarded. Plates were then stained with neutral red or crystal violet, washed, and the number of viable fibrosarcoma colonies of more than 20 cells counted. Lymphoid cells did not form colonies.

Results and Discussion. The experimental results are summarized in Tables I and II. Lymph node cells from immune rats were found to be capable of reducing the plating efficiency of tumor cells to 67 and 70% of

TABLE I. Effect of Syngeneic Lymphoid Cells on Plating Efficiency of BNSE3049 Tumor Cells.

Experiment[a]	Treatment of tumor cells	Number of colonies	Mean number of colonies	Per cent of control plating efficiency
I C	Control	22,24	23	100
	Normal lymph node cells	20,24	22	96
	Normal spleen cells	23,25	24	104
	Immune lymph node cells	17,14	15.5	67
	Immune spleen cells	19,17	18	78
II C	Control	28,37,29,36,23,38,30, 31,22,36,32,27,32,28, 37,34,33,38,38,28,22	31.4	100
	Normal lymph node cells	26,26,25,26,29,19,26 32,34,19	26.2	83
	Immune lymph node cells	17,15,22,29,19,21,32	22.1	70

[a] The number of tumor cells plated in Expt. I C was 100 and the plating efficiency of the control was 23%; in Expt. II C, 50 tumor cells were plated and the plating efficiency was 62.8%.

TABLE II. Effect of Serum on the Plating Efficiency of BNSE3049 Tumor Cells.

Experiment[a]	Treatment of tumor cells	Number of colonies	Mean number of colonies	Per cent of control plating efficiency
	Control	67,68,87,64,53,60,65, 53,60	64	100
	Complement, 0.1 ml, undiluted	0,0,14	5	8
	0.1 ml of 1/10 complement (used in subsequent experiments)	66,58	62	97
I S	Control	17,10,10,24,30,31,13	19	100
	Complement	33,12,12	19	100
	Lewis anti-BNSE3049	0,15,12	9	47
	Lewis anti-BNSE3049 + complement	0,0,1	0	0
II S	Control	29,26,30,25,23,26	26.5	100
	Complement	25,21,23,29	24.5	92
	Tumor-bearing BN	23,26,26,28	25.75	97
	Tumor-bearing BN + complement	19,19,20	19.3	73
III S	Complement (no untreated control)	23,24,21	22.7	100
	Normal BN + complement	21,25,21	22.3	98
	Lewis anti-BNSE3049 + complement	10,13,12	11.7	52
	Tumor-bearing BN (heat inactivated)	24,21	22.5	99
	Tumor-bearing BN (heat inactivated) + complement	21,19,14,18	18.0	79
IV S	Control	24,34	29	100
	Normal BN	24,37,28	29.6	102
	Normal BN + complement	22,35,30	29	100
	Immune BN	28,14,40	27.3	94
	Immune BN + complement	34,33,25	30.6	106

[a] The number of tumor cells plated in Expt. I S was 100 and the control plating efficiency was 19%; in Expt. II S, 50 cells were plated and the control plating efficiency was 53%; in Expt. III S, 62 cells were plated and the control plating efficiency was 36.6%; in Expt. IV S, 100 cells were plated and the control plating efficiency was 29%.

the control level. Immune spleen cells were slightly less effective. Serum from immune rats was effective in reducing the plating efficiency only if produced in allogeneic animals. Syngeneic rats, immune to the polyoma-induced tumor, contained no serum factor that reduced tumor growth *in vitro*. Serum from tumor-bearing rats contained a factor which was stable at 56° and in the presence of rabbit complement reduced the plating efficiency of the tumor cells. In these experiments plating efficiencies of up to 63% were obtained in control cultures. The high control plating efficiency allows one to avoid the problem of having to infer an effect of treatment on the behavior of a large number of tumor cells from data based on a few cells that conceivably could form colonies under control conditions.

In interpreting the data relating to cellular immunity, it is worthwhile to mention that care was taken to avoid exposing the rats to be immunized to fetal calf serum. For this reason, the cloned tumor cells were first carried for several transplant generations in syngeneic rats before this tumor was implanted in the footpad of the animals that would provide the lymphoid cells. This manipulation avoided a nonspecific cytotoxic effect on the tumor secondary to an immune interaction between sensitized lymphoid cells and a protein antigen in the medium (15).

Other workers have reported an effect of lymphoid cells and serum from immune mice on the plating efficiency of a syngeneic lymphoma cell line (16) carrying the polyoma antigen (9, 10). The fibrosarcoma used in the present study was susceptible to Lewis anti-BN serum; however, this effect was achieved only with high concentration of serum. Thus, immune serum in the presence of complement could influence plating efficiency of the tumor cells. When the same amount of serum from tumor-immune syngeneic rats was used, however, no effect was obtained on the plating efficiency. Whether this represents a decreased number of polyoma antigenic sites compared to histocompatibility sites or an absence of humoral antitumor activity cannot be resolved by these experiments. There is suggestive evidence that the serum factors present in the tumor-bearing rats represent specific antibody. The factor is stable at 56° and complement is required for activity. It is of interest that this serum from immune rats did not react with tumor cells when tested by indirect immunofluorescence. Whether this factor was specifically directed against the growing tumor or represented some nonspecific toxic factor cannot be resolved by the present experiment.

These results point to the essential role of lymphoid cells in resistance in rats immune to polyoma-induced fibrosarcomas. Presumably the same cell–tumor interaction, impaired by thymectomy and restored by passive transfer of lymphoid cells, would be effective in host protection during tumor induction by polyoma virus.

Summary. An *in vitro* technique for plating tumor cells with a high efficiency is described. BN rats were immunized to a syngeneic, cloned, tissue culture-adapted, polyoma-induced fibrosarcoma (BN SE3049) by amputation of a tumor-bearing extremity. Lymphoid cells from spleen and draining lymph nodes, but not serum, from these rats were effective in reducing the plating efficiency of the tumor. This finding is consistent with the necessary role of cellular immunity in resistance to polyoma oncogenesis.

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