

Effect of modified tumor antigen on experimentally induced sarcomas

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ABSTRACT

Objective: To explore the possibility of controlling established tumors by active immunization through specific and nonspecific methods.

Methods: By subcutaneous methylcholanthrene, a fibrosarcoma was produced in adult Swiss male mice. The fibrosarcoma was transplanted into the isogenic strain. The cleared tumor cells were injected subcutaneous into the hind leg of the 1st sarcoma group. The 2nd group received intraperitoneally sensitized spleen cells. One section of the irradiated 3rd tumor group received intraperitoneally sensitized spleen cells and subsequently a mild dose of modified tumor antigen. The 2nd section of the 3rd group was irradiated in between the administration of modified tumor antigen. In both the groups, liver of normal and transplanted tumor bearing mice was processed and intraperitoneally injected into the isogenic tumor bearing mice. Histopathology and tumor size by calipers was assessed.

Results: The first group showed enhancement of tumor growth in all its 3 fractions injected at different intervals.

In the 2nd group, the average survival period was prolonged. In the first section of the 3rd group a decrease in tumor size and protracted survival was noted. In the transplanted tumor bearing mice, complete suppression of tumor growth was observed. In the 2nd fraction of the 3rd group, long survival period with regression of tumor was observed. In the 4th group, attenuated tumor and one mouse was observed to become tumor free after 60 days.

Conclusions: Active immunosuppression by sensitized spleen cells and vaccines from transplanted sarcomas enabled regression of tumor size and longevity in tumor bearing mice. The modified tumor antigens, processed isogenic liver cells attenuated to zero level in tumor size in isogenic transplanted tumor bearing mice. The results show vaccines from allogenic and syngeneic sources bear the potential to regress tumor and enhance survival period.

Keywords: Modified tumor antigen, experimental studies.

Saudi Medical Journal 2001; Vol. 22 (2): 157-163

The C₃H mice bearing either spontaneous mammary adenocarcinoma or methylcholanthrene (MC) induced squamous cell carcinoma could be immunized against their own tumor.¹ This was achieved by modifying the surface properties of tumor cells obtained from biopsied samples by coupling with tumor globulin through bisdiazobenzidine (BDB). In addition to retardation of growth and extensive necrosis of tumor, cells are like a mammary tumor. With virus induced tumor in

situ, or as heterotropic grafts, the injection of an irradiated or freeze thawed preparation of a biopsy sample of the liver tumor host interfered with the growth of the subcutaneous tumor. The object of this study was to explore the possibility of controlling the established tumor by active immunization² and also using non-specific methods to enhance the immunologic reactivity of the tumor bearing mice. This is a pilot study on primary and transplanted tumors.

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Received 14th September 2000. Accepted for publication in final form 26th September 2000.

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Methods. Adult Swiss, Virus Research Center, (VRC) India mice weighing on an average 20-25 gms were employed in this study. Average age of the animals varied from 2 to 3 months. The animals were kept on a standard balanced diet prepared in the laboratory according to formula prescribed by the National Institute of Nutrition, Hyderabad. Water was given ad libitum. One mg of 20 MC was mixed thoroughly in 0.1 cc of olive oil and injected subcutaneous (*sc*) (with a 27-G needle) above the scapular region. Fibrosarcomas developed in the region in 80-120 days (Figure 1). Fibrosarcomas so produced were transplanted into the isogenic strain.

Preparation of Bisdiazobenzidine. Bisdiazobenzidine was prepared as follows: Two solutions of benzidine 0.46% and nanoz 0.35% were mixed and stirred continuously by a magnetic stirrer for 30 minutes at 7-8°C. The aliquot was quickly frozen in a dry ice alcohol bath and stored at -20°C. This aliquot was thawed and diluted to 1:15 using 0.2 gm phosphate buffer (pH 7.3) for use.

Preparation of tumor antigen. After tumor is histologically confirmed, 0.5gm of the tumor was removed and in Hanks balanced salt solution with antibiotics, freed from blood vessels, necrotic material and fibrous tissues. A suspension of tumor cell was prepared by cutting into fine pieces and breaking the tumor cell with a magnetic stirrer at 4°C. The residue was passed through a sieve with a porosity of approximately 100 μ . All the tumor cells were collected and centrifuged at 500 RPM for 10 minutes and the supernatant discarded. The cells were re-suspended in 1.5 ml of 0.9% sodium chloride (NaCl). This was allowed to act with 2 ml of solution of fraction II rabbit gamma globulin (1 mg/ml) in 0.9% NaCl. These were acted by a freshly prepared 1:15 BDB in phosphate buffer and allowed to remain at room temperature for 15 minutes, centrifuged and the supernatant discarded. After washing the cells twice with normal saline solutions, these were suspended in 0.5 ml of 0.9% NaCl and mixed thoroughly with 1 ml of complete Freund's adjuvant (FA). The animals bearing tumor were given isologous and homologous 0.25 ml of modified tumor cell antigens at intervals of 3 weeks and 2 weeks and thereafter until time of sacrifice.

Injection in animals. Group I. In one hind leg thigh of each animal 0.25 ml of tumor foreign protein complex in complete FA was injected. The control animals were injected with a suspension of tumor cells in complete FA.

Group II. Sensitized spleen collected from transplanted tumor bearing mice. Spleen cell suspensions were prepared³ and then diluted to a concentration of 10^9 cells per 0.4 cc and then injected intraperitoneally (*ip*) into the group of mice bearing transplanted tumor. Before this injection, tumors were measured by calipers, and again after the 4th day the same dose was repeated. On the 10th day

modified cell antigens were started and the results noted later.

Group III. Tumor bearing mice of 2 weeks duration were further subdivided into 2 groups. In one group whole body X-irradiation of 400 R was followed 24 hours later by 10^9 sensitized spleen cells *ip* and after one weeks gap, modified tumor antigen (MTA) cells were given. In the 2nd group, one injection of MTA, as given earlier, followed a week later by whole body irradiation of 400 R and then subsequently after a further weeks interval the vaccine was repeated.²

Group IV. Here, we employed the technique with a slight modification.⁴ Liver tissue from the isogenic strain; bearing transplanted tumor was removed aseptically and was divided into small pieces. The small pieces were minced separately with the help of a scissor in Hanks balanced salt solution. An aliquot of 20-25 mg of liver was kept separately in Bijou bottles at -20°C. Similarly, normal liver tissue was prepared. In both the cases tumor cells in 10^3 numbers were mixed with liver homogenate. The combined liver and tumor tissue was frozen and thawed 5 times in succession. The cells were resuspended in 0.2 ml normal saline. The initial inoculation of vaccine material was mixed with 0.2cc of complete FA and injected into the isogenic tumor bearing mice. The same method was followed for normal liver tissue.

Histology. The tissue collected from the tumors at the end of the experiment were preserved in 10% formaldehyde, serial sections were cut and stained with Ehrlich's hematoxylin and eosin.⁵

Measurement of the tumor. The tumors were measured in 2 planes by calipers and the tumor size calculated.⁶

Results. Gross observations of tumor growth.

Gross measurements (by calculating the size of the tumor) were taken to assess tumor growth at the beginning and end of the study period. The average size of the tumor initially and the final average size of the tumor was considered to be a measure of absolute growth. Percentage of surviving animals and survival time in days of individual mice was also considered. This data was then plotted on a graph and in histogram form (Figure 2). In the first experiment, attempts were made to vaccinate the 20 MC injected mice at different time intervals. The first group was given vaccine prepared from homologous tumor tissue for one month, the 2nd group 2nd month and 3rd group 3rd month after the administration of 20 MC. The results were recorded on the 90th, 104th and 120th days after 20 MC injection. It was observed (Figure 3) that there was an enhancement of tumor growth in all the 3 vaccinated groups particularly in the 3rd group. The results however were statistically significant. Subsequently, the modified pool of tumor cell vaccine was tried on



Figure 1 - Mouse showing 20 methyl cholanthrene induced primary tumors.

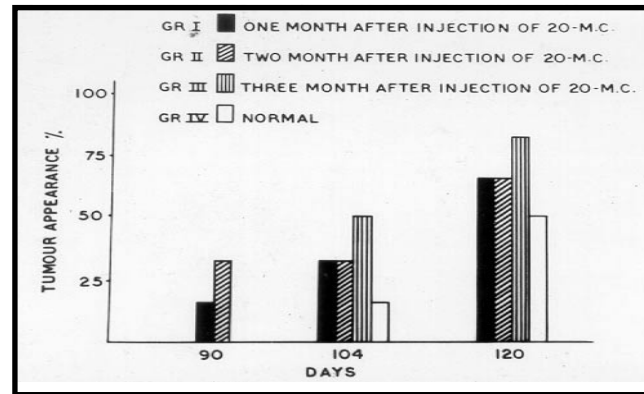


Figure 2 - Effect of modified tumor antigen vaccine administered prior to the production of primary tumors. MC - Methylcholanthrene; GR - Group.

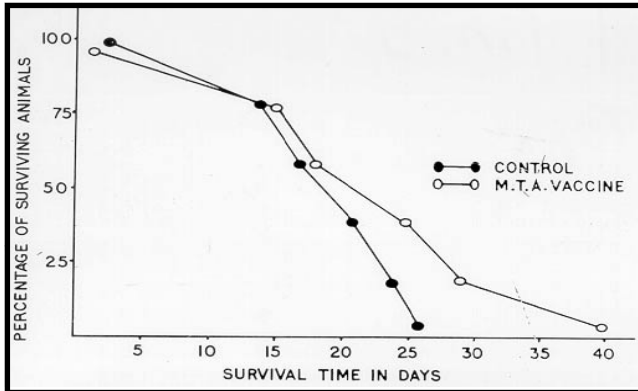


Figure 3 - Effect of pooled tumor cell vaccine on primary tumor bearing mice. MTA - Modified tumor antigen.

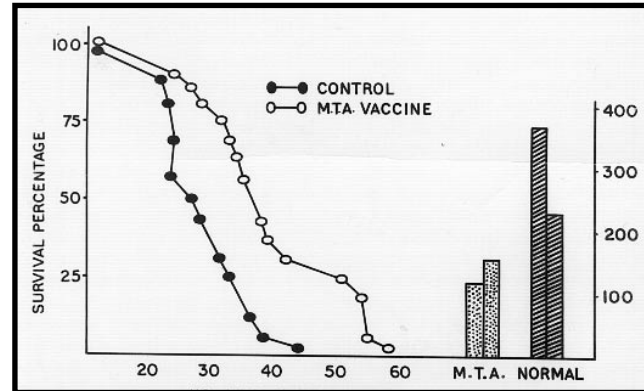


Figure 4 - Effect of modified tumor antigen (MTA) vaccine on transplanted tumors.

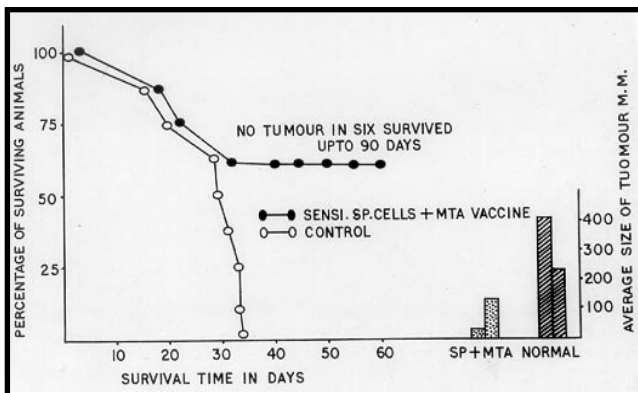


Figure 5 - Effect of intraperitoneally administration of sensitized spleen cells (sensi. sp. cells) and modified tumor antigen (MTA).

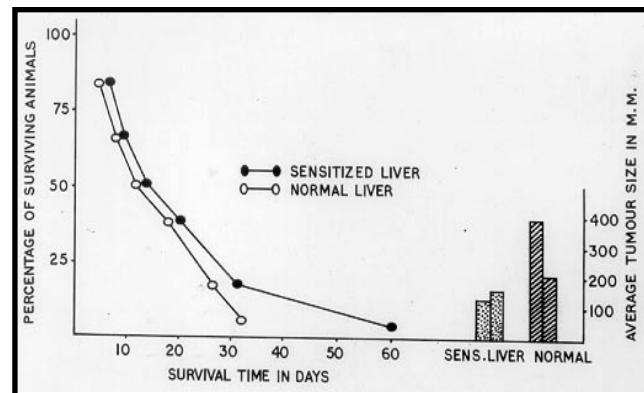


Figure 6 - Effect of isogenic sensitized liver (sens. liver) tissue plus tumor cells and complete Freund's adjuvant on transplanted tumors.

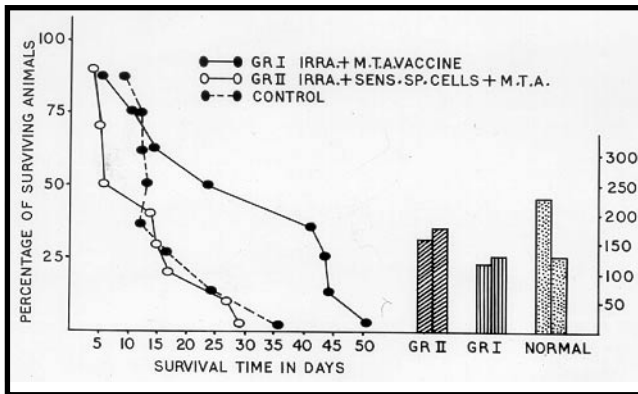


Figure 7 - Combined effect of irradiation (IRR) plus sensitized spleen (Sens. SP.) cells and modified tumor antigen (MTA) on tumor transplant. GR - group.

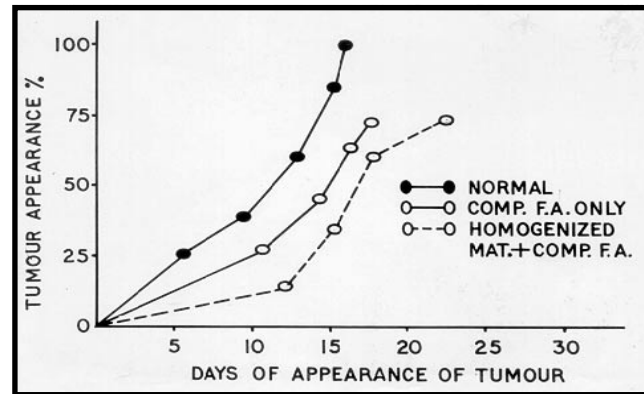


Figure 8 - Effect of homogenized cell free material and complete Freund's adjuvant (Comp. F.A.) on transplantable tumors. MTA - modified tumor antigen.

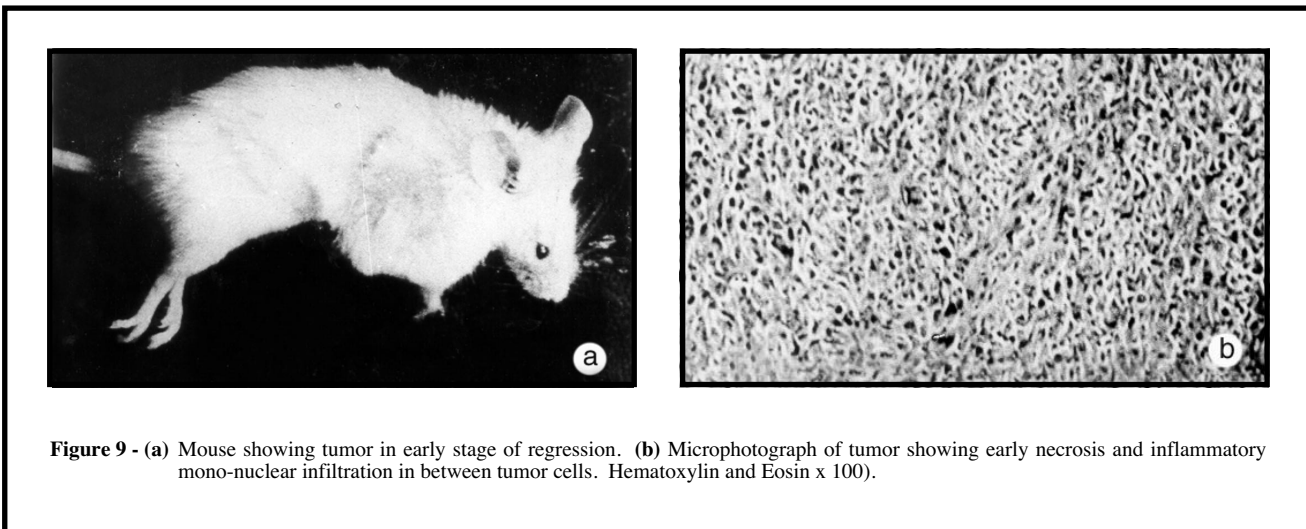


Figure 9 - (a) Mouse showing tumor in early stage of regression. (b) Microphotograph of tumor showing early necrosis and inflammatory mono-nuclear infiltration in between tumor cells. Hematoxylin and Eosin x 100).

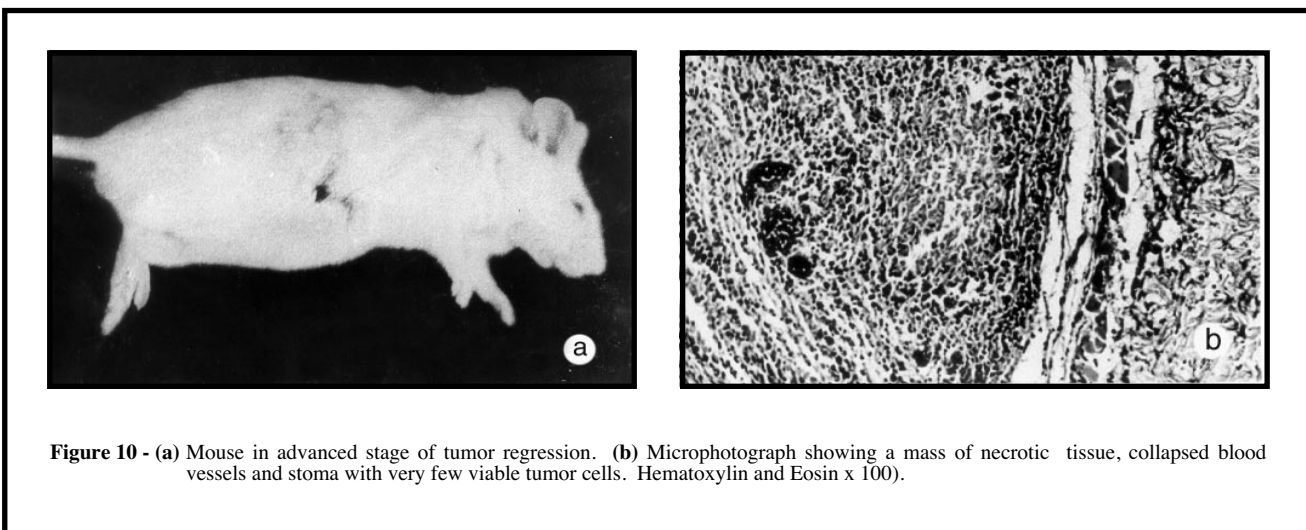


Figure 10 - (a) Mouse in advanced stage of tumor regression. (b) Microphotograph showing a mass of necrotic tissue, collapsed blood vessels and stroma with very few viable tumor cells. Hematoxylin and Eosin x 100).

primary tumor bearing mice, which had comparatively well established tumors. Three injections of the vaccine prepared from pooled homologous tumor tissue were given at different time intervals. It was observed that after 2nd injection there was a reduction in the growth potential of tumor. The average survival time was also increased in the vaccinated group as compared to the control group. Statistical analysis did not reveal any significant results (Figure 4).

Effect of modified tumor antigen vaccine on transplanted tumor. Transplanted tumors of 3rd passage were utilized for this study. Injections of MTA vaccine were administered at different time intervals and the tumor size was measured with the help of vernier calipers. Survival period of individual mice was recorded and was compared with the control group. It was observed that there was a highly significant increase in the period of survival of mice in the vaccinated group as compared to the control group. In the majority of animals, the tumor size had decreased and the tumor felt soft and pulpy. However, the results of measurements of tumors were not significant (Figure 5).

Combined effect of modified tumor antigen vaccine and sensitized spleen cells. The transplanted tumor bearing mice of 4th passage were utilized. The sensitized spleen cells were collected from the isogenic group of mice and 10^9 sensitized spleen cells were *ip* injected into the transplanted tumor bearing mice. The size of the tumor was measured. The same dose was repeatedly again on the 4th day. After 7 days MTA vaccine was injected. It was observed that after the first injection of the MTA vaccine there was a complete suppression of tumor growth in 6 mice. Six out of 8 mice were observed to survive without tumor for 90 days. The results are presented in Figure 6 which shows the effect of administration of sensitized spleen cells followed by MTA vaccine on transplanted tumor.

Effects of isogenic liver cells. Isogenic liver cells 15-20 mg were minced, frozen and thawed 5 times and mixed with 10^3 tumor cells and emulsified with 0.2cc of complete FA and administered to isogenic transplanted tumor bearing mice of 5th passage. After the 2nd injection was repeated, the size of the tumor decreased significantly ($p < 0.05$) and one mouse became tumor free for 60 days. The results are shown in Figure 7, which shows the effect of isogenic liver tissue, and tumor cells and complete FA on transplanted tumors.

Effect of modified tumor vaccine and total body irradiation. Modified tumor antigen vaccine was injected first and followed a week later by 400 R whole body irradiation. Modified tumor antigen vaccine was repeated after irradiation. There was an increase in the survival time as compared to the control, and also there was regression in tumor size but the results were not statistically significant.

The combined effect of whole body irradiation. Modified tumor antigen vaccine and sensitized spleen cells. In the 2nd group after recording the size of the tumor, they were subjected to whole body irradiation of 400 R followed 24 hours later with injection of 10^9 sensitized spleen cells *ip*. After a one week gap, the MTA vaccine was administered. There was a considerable graft versus host reaction and there was a sharp rise of mortality from the 3rd day onwards. The injection of MTA was administered afterwards in surviving mice. It was noted that there was a decrease in the average survival time of mice as compared to the control group. However, the tumor size became static after the treatment with sensitized spleen cells. The results have been presented (Figure 8).

Effect of cell free tumor material and Freund's adjuvant. Four injections of homogenized cell free materials from isogenic tumor cell were mixed with complete FA and administered to a group. The control group received 0.2 ml of FA. The animals were challenged with 10^5 viable tumor cells against which they were immunized. No significant difference was observed between the 2 groups.

Discussion. In allogenic as well as syngenic relationships, immune acceleration of tumor growth after active immunization was often observed.⁷ In the present study; the animals were divided into 3 groups. In group I the vaccine was administered for one month and Grade II for 2 months and Grade III, 3 months after the injection of 20 MC. Three injections were given in each group. It was observed that tumor appeared earlier in the vaccinated group when compared to the control group. This effect was more marked in the 3rd group. It is possible that at the dosage used, pooled modified tumor antigenic cells in complete FA are incapable of inducing detectable levels of enhancing antibodies. They sensitized the host, so that the subsequent appearance of primary tumor evoked a humoral rather than cellular amnestic response. These humoral antibodies blocked the antigenic sites in tumor cells rendering them safe from the attack of cell mediated immunity.⁴ Attempts¹ were made to modify the tumor cells and making them more antigenic by coupling the cells with a highly antigenic protein utilizing BDB as a coupling agent. In the present study, pooled tumor cells were used and found that the cells were tagged with foreign protein complex in the presence of the BDB. A decrease in the size of the tumor and an increase in the average survival period were seen. (Figure 9a and 9b). It is postulated that pooled tumor cells carry cross reacting antigens to the primary tumor⁸ and these minor antigenic alterations could help in sensitizing the animal leading ultimately to restricted growth of the tumor. When similar studies were undertaken with

transplanted tumor using isogenic tumor cells of the same passage the cell antigenicity was modified as has been described earlier.⁴ The MTA complex with complete FA was injected into a group of transplanted tumor bearing mice, it was observed that there was a significant increase ($p < 0.01$) in the average survival period of mice when compared with the control. It has been shown,⁹ that the effect of sensitized spleen cells in leukemia and neuroblastomas, the latter by using colony inhibition test, and the animals receiving sensitized syngeneic lymphoid cells remained free of tumor throughout their life.⁷ In our own experiment, it was observed that when sensitized spleen cells were administered intravenously they caused a marked increase in the survival period of mice with a considerable decrease in size of the tumor when MTA vaccine was combined with sensitized spleen cells given *ip*. A complete suppression of tumor growth was observed in 75% of the mice and the tumor free mice survived up to 90 days. The results were highly significant ($p < 0.01$) when compared with the control and with sensitized spleen cells alone. This combination has been used for the first time in experimental studies and the results indicate the enhancement of a tumor suppressive effect when the vaccine is combined with sensitized lymphocytes from the syngeneic strain. In various materials from the syngeneic tumor bearing mice, it was found that syngeneic liver tissue with complete FA caused a significant reduction in the size of the tumor and the period of survival was increased. The present study was undertaken to determine the usefulness of isogenic liver cells as a vaccine. Isogenic liver tissue and tumor cells with complete FA were injected to transplanted tumor bearing mice, a significant ($p < 0.01$) reduction in the size of the tumor was seen (Figure 10a and 10b). It has also been postulated that the majority of the circulating tumor antigens are taken up by Kupffer cells. The tumor was greatly retarded by whole body irradiation combined with *ip* injection of thoracic duct lymphocyte from rats immunized against the tumor. These mice subsequently died of graft versus host reaction. It was also observed that auto-immunization with primary tumor may not result only in regression, but also increase the irradiation sensitivity.¹⁰ In the present study, whole body irradiation was combined with *ip* injection of isogenic sensitized spleen cells and later MTA vaccine. A heavy mortality was observed on the other hand, when MTA vaccine was administered earlier followed a week later by whole body irradiation, there was a considerable reduction in the size of the tumor and the mean survival period was also considerably increased.

Three types of cell free extracts both in, observed active immunization and increased resistance to tumor transplants, particularly in the group where

saline tumor extract with complete FA, did not produce any significant effect on similar experiments.¹¹ It is possible that the tumor cell used for immunization may be carrying a very weak antigen and was not able to produce active anti-tumor immunity.¹² As it appeared, the maximum effect was recorded in the MTA vaccine group and MTA and sensitized spleen cell group. When Group II and III were compared, there was a statistically significant increase in survival period of mice in Group III ($p < 0.01$), similarly when Group III was compared with Group 1, 4, 5 and 6. The results were highly significant with Group 5 ($p < 0.01$) and significant with 1, 4 and 6 ($p < 0.05$). With Group II, when a similar comparison was made, the average survival period was only significant ($p < 0.05$). These results suggest that the MTA was effective in increasing the period of survival of mice when compared to Group 1, 4, 5 and 6. However, when spleen cells were combined with MTA vaccine the results were highly significant when compared to Group II. This indicates the enhancing effect of sensitized lymphocytes when combined with MTA vaccine.

The antigenicity of tumor cells was enhanced by coupling them with (Cohn fraction II rabbit gamma globulin) in the presence of BDB, and when 20 MC inoculated mice were vaccinated with pooled modified tumor cells antigen vaccine, one month, 2 months and 3 months after the administration of the carcinogen. There was an apparent enhancement of tumor growth which was however not statistically significant, when mice bearing primary MC induced tumors or transplanted tumors were actively immunized by MTAs, a reduction of tumor growth with an increase in the average survival period was observed in primary tumor bearing mice. On the other hand, there was a significant increase in the survival period of mice with transplanted tumor. In sensitized spleen cells, when administered *ip* in mice with transplanted tumors and the dose was repeated after 4 days followed by the MTA vaccine, a significant reduction in tumor size was obtained and in 75% of the animals there was a complete regression of tumors. When modified tumor vaccine was administered with whole body irradiation, the results were not significant when compared with the control. A statistical analysis of this result indicates that maximum tumor suppressive effect was observed with MTA, and when MTA was combined with sensitized spleen cells, there was a statistically significant suppression of tumor when these 2 groups were compared.

Immunization with 'freeze thawed' isogenic sensitized liver tissue when combined with lower doses of tumor cells and complete FA, interfered significantly with the growth of the isogenic transplanted tumor. The liver homogenate from normal animals was totally ineffective. It is

suggested that the sensitized liver tissue may be carrying activated tumor antigen in the Kupffer cells. When mice were immunized with cell free material and were subsequently challenged with viable tumor cells, no significant effect was observed.

Acknowledgment. The authors thank the Director, National Institute of Occupational Health, Ahmedabad, India for encouragement and facilities.

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