

## Effect of "Rasayanas" a herbal drug preparation on cell-mediated immune responses in tumour bearing mice

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Administration of herbal preparation, Rasayanas has been found to enhance the natural killer cell activity in normal as well as in tumour bearing animals. Brahma Rasayana (BR) was found to have the maximum activity. BR and Ashwagandha Rasayana (AR) were found to activate antibody-dependent cellular cytotoxicity significantly. AR was also found to activate macrophages. All the Rasayanas were found to stimulate antibody dependent complement mediated tumour cell lysis. The results of these studies indicate usefulness of Rasayanas for immunostimulation in normal and in disease state.

Rasayanas are a group of non-toxic herbal drug preparations which are used to improve the general health. It is believed that Rasayanas mainly act by stimulating the body's immunity. However, no concrete studies were done to substantiate these claims. Previously we have shown that some of the Rasayanas could increase the total count of leukocytes and percentage of neutrophils in mice<sup>2</sup>. Rasayanas were found to protect the mice from cyclophosphamide<sup>3</sup> and radiation<sup>4</sup> induced myelosuppression. In the present manuscript we demonstrate the stimulatory activity of Rasayanas on the cellular effector mechanism against tumour cells.

### Materials and Methods

Brahma Rasayana (BR), Narasimha Rasayana (NR), Ashwagandha Rasayana (AR) and Amrutha Prasham (AP) were purchased from "Vaidyaratnam Oushadhasala", Ollur. The main ingredients of these Rasayanas were described elsewhere<sup>2</sup>.

YAC-1 cells and L-929 were obtained from National Facility for Animal Cells and Tissue Culture, Pune and Ehrlich ascites tumour cells were originally obtained from Cancer Research Institute, Mumbai. Sheep red blood cells (SRBC) were freshly collected in Alsever's solution. Balb/c mice (6-8 weeks) were purchased from National Institute of Nutrition, Hyderabad, India.

The mice were divided into four groups. Group I-normal animals, group II-tumour alone, group III-normal animals treated with Rasayanas and group IV-tumour bearing animals treated with Rasayanas. Rasayanas (50 mg/animal/dose) were given daily orally for a total of 5 doses for all the experiments. Along with the last dose of Rasayana treatment, Ehrlich ascites tumour cells ( $10^6$ ) were injected (ip) to groups II and IV. At various time intervals the animals were sacrificed and a single cell suspension of whole spleen cells were made and used as effector cells. No attempt was made to remove adherent monocyte or macrophage population.

Target cells were labelled by incubating YAC-1 cells ( $1 \times 10^6$ ) or SRBC ( $1 \times 10^7$ ) with  $\text{Na}_2^{51}\text{CrO}_4$  (100  $\mu\text{Ci}$ ) (BRIT, Bombay) at  $37^\circ$  for 1 hr. The cells were washed and suspended at a concentration of  $1 \times 10^5$  cells/ml. Anti-SRBC antibody was raised in rabbits and was used as a source of antibody for ADCC assay.

*Assay for natural killer cell activity*—A short term (4 hr)  $^{51}\text{Cr}$ -release assay in triplicates was performed in RIA tubes for the determination of NK-cell activity. The labelled target YAC-1 cells ( $1 \times 10^4$  cells/tube), specific to NK-cell, were incubated with spleen cells at an effector target ratios of 100:1, 50:1, and 25:1 at  $37^\circ\text{C}$  for 4 hr. After the incubation the tubes were centrifuged and an aliquot of the supernatant was counted for radioactivity released after cell death using a gamma ray spectrometer.

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**Determination of antibody-dependent cellular cytotoxic activity**—ADCC activity was determined<sup>7</sup> using chromium labelled SRBC as targets and whole spleen cells population as effector cells. SRBC ( $1 \times 10^4$  cells/tube) were incubated with spleen cells at different effector:target ratios of 100:1, 50:1 and 25:1 along with 0.1 ml of anti-SRBC antibody in a total volume of 0.4 ml at 37° C for 4hr. The tubes were centrifuged and 0.1 ml of the supernatant was counted for released radioactivity as above.

**Antibody-dependent complement-mediated lysis**—Blood was collected from the animals from all groups on various days, sera separated and heat inactivated at 56°C for 30 min. Fresh rabbit serum diluted with RPMI-1640 was used as a source of complement. To serially diluted sera samples 0.1 ml of Ehrlich ascites tumour cells ( $1 \times 10^4$  cells) and 50 µl of complement were added. The final volume was made up to 2 ml and incubated at 37°C for 3hr. Samples with cells alone and cells treated with complement and cells treated with antibody were kept as controls. The cytotoxicity was assessed 6 by trypan blue exclusion method. All the tests were done in triplicates. Antibody-dependent complement mediated lysis was calculated from the formula

$$\% \text{ dead cells} = \frac{\text{No. of dead cells - No. of dead cells with complement only}}{\text{No. of viable cells + No. of dead cells}} \times 10$$

**Assessment of macrophage-mediated tumour cell lysis**—Peritoneal macrophage mediated cytotoxicity was determined according to the method of Flick *et al.*<sup>8</sup>. Different groups of mice were treated with five doses of Rasayanas and at the end of the treatment 0.2 ml of 5% sodium caseinate was injected (ip) to induce macrophages. On 5th day the animals were sacrificed and the macrophages were collected, washed and suspended in DMEM containing 10% FCS at a concentration of  $2.5 \times 10^6$  cells/ml.

L-929 cells ( $5 \times 10^3$  cells/well) were added to 96-well plates and incubated at 37° C in a humidified atmosphere containing 5% CO<sub>2</sub> for 24hr. Macrophages ( $2.5 \times 10^4$  cells/well) from each group were added to each well (in quadruplicates) and incubated at 37° C in a humidified atmosphere containing 5% CO<sub>2</sub> for 48hr. The cells were washed, fixed in 0.5% formalin and stained with 0.05% crystal violet. The cell death was assessed

by checking the morphology using an inverted microscope. The cells were lysed in water and the interacting of staining which is measure of cell growth was measured using an Elisa plate reader at 650 nm.

## Results

**Effect of Rasayanas on natural killer cell activity**—The effect of various Rasayanas on NK-cell mediated cytotoxicity in different groups of animals is shown in Table 1. Oral administration of Rasayanas augmented the NK-cell activity in tumour-bearing animals which occurred early when compared to untreated tumour-bearing animals. In control tumour-bearing animals the peak lysis (48%) was observed on day 9. Among the four Rasayanas studied the activity of AR was found to be superior and it produced 43% target cell lysis as early as day 2. Administration of Rasayanas also enhanced NK-cell activity in normal animals. In this case also an early appearance of activity (day 2) was seen in AR treated groups with target cell lysis of 41%. However percent lysis did not significantly alter.

**Effect of Rasayanas on antibody-dependent cellular cytotoxicity**—Effect of oral administration of Rasayanas on ADCC in various groups of animals is represented in Table 2. Among the four Rasayanas studied only AR and BR were found to enhance the ADCC 43% and 58% lysis respectively at day 5 earlier in tumour-bearing animals. In tumour-bearing control animals the peak activity was on day 7 with target cell lysis of 50%. All the Rasayanas were also found to augment ADCC in normal animals.

### Effect of Rasayanas on antibody-dependent

Table 1—Effect of Rasayanas on NK-cell activity in mice

| Treatment          | Day on which peak lysis was observed** | % target lysis* |
|--------------------|----------------------------------------|-----------------|
| Untreated controls | -                                      | 5               |
| Tumour alone       | 9                                      | 48              |
| BR alone           | 7                                      | 59              |
| BR+ Tumour         | 7                                      | 55              |
| NR alone           | 5                                      | 50              |
| NR+ Tumour         | 3                                      | 43              |
| AP alone           | 5                                      | 48              |
| AP+ Tumour         | 5                                      | 43              |
| AR alone           | 2                                      | 41              |
| AR+ Tumour         | 2                                      | 43              |

\* The values given are at an effector:target ratio of 100:1

\*\* Five doses of Rasayanas were given prior to the determination of NK cell activity and tumour cells were injected along with the last dose of drug treatment.

Table 2—Effect of Rasayanas on ADCC activity in mice

| Treatment        | Day on which peak lysis was observed** | % target lysis* |
|------------------|----------------------------------------|-----------------|
| Without Rasayana | -                                      | 7               |
| Tumour alone     | 7                                      | 50              |
| BR alone         | 5                                      | 61              |
| BR+ Tumour       | 5                                      | 58              |
| NR alone         | 7                                      | 59              |
| NR+ Tumour       | 7                                      | 46              |
| AP alone         | 7                                      | 60              |
| AP+ Tumour       | 7                                      | 57              |
| AR alone         | 5                                      | 52              |
| AR+ Tumour       | 5                                      | 43              |

\* The values given are at an effector:target ratio of 100:1

\*\* Five doses of Rasayanas were given prior to the determination of ADCC activity and tumour cells were injected along with the last dose of drug treatment.

Table 3—Effect of Rasayanas on NK-cell activity in mice

| Treatment    | Day on which maximum cell death was observed | % cell death |
|--------------|----------------------------------------------|--------------|
| Tumour alone | 21                                           | 19           |
| BR+Tumour    | 15                                           | 22           |
| NR+Tumour    | 17                                           | 31           |
| AR+Tumour    | 15                                           | 24           |
| AP+Tumour    | 15                                           | 38           |

5 doses of each Rasayana were given prior to the tumour inoculation and 24hr after the inoculation sera were collected on various days to perform the assay.

*complement-mediated cytotoxicity in tumour-bearing animals*—The effect of Rasayanas on ACC in tumour-bearing animals is shown in Table 3. All Rasayanas enhanced ACC in the tumour-bearing animals. The maximum activity was observed on day 15 in the case of BR, AR and AP and for NR on day 17. In the case of tumour-bearing control animals the maximum cytotoxicity was observed on day 21. Percentage lysis did not say significantly.

*Effect of Rasayanas on macrophage activation*—Among the four Rasayanas studied only AR was found to activate macrophages (Figs 1A, B). Normal peritoneal macrophages induced by sodium caseinate showed no lysis of tumour cells (Fig. 1A). But macrophages isolated from AR treated which substantiated by morphology as well as the measurement of the stained cells animals showed complete lysis of L-929 cells (Fig.1 B). which was substantiated by morphology and measurement of stained cell extracts.

## Discussion

The present report demonstrated the activation of immune effector mechanisms against tumour

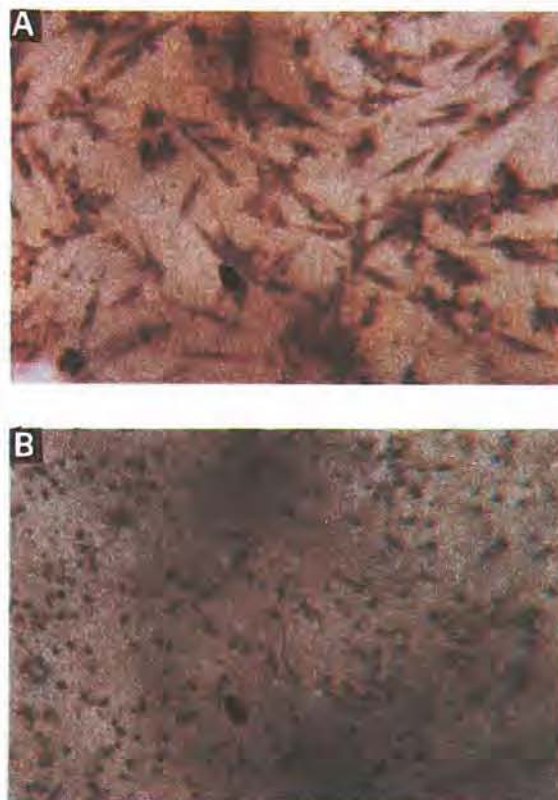


Fig. 1—Effect of AR on macrophage activation. (A) L-929 cells treated with macrophages from animals treated with sodium caseinate alone and (B) L-929 cells treated with macrophages from animals treated with sodium caseinate and AR.

cells after Rasayana treatment. Administration of Rasayanas enhanced the activity of non-specific immune cells such as NK-cells and macrophages. It has been shown that NK-cells lack both T-cell and B-cell markers and require no prior sensitization to cause target cell lysis<sup>9</sup>. They play an important role in cancer cell invasion and metastasis<sup>10</sup>. Rasayana treatment enhanced the NK-cells in normal animals and tumour-bearing animals. As the tumour cells could be destroyed before the tumour load is too large, an early activation of NK cells is beneficial to the host during cancer development. Administration of AR was found to activate the macrophages which were highly cytotoxic to L-929 cells. As L-929 cells are highly sensitive to tumour necrosis factor (TNF)<sup>11</sup> mechanism of tumour cell lysis may be due to the TNF released by macrophages.

Rasayana treatment was found to enhance the ADCC activity in tumour-bearing animals and in normal animals. Rasayanas also activated ACC in tumour-bearing animals. We have already shown

that Rasayanas could increase the life span of ascites tumour-bearing animals and reduce the solid tumour volume induced by Daltons lymphoma ascites tumour cells<sup>2</sup>. As Rasayanas were found not to have direct cytotoxic effect, the tumour regression seen may be due to the increased immunostimulation after administration of Rasayanas.

A herbal mixture, similar to Rasayanas, Mahsarishi Amrit Kalash (MAK-4) have been shown to have several biological properties. It has been shown to have antioxidant properties<sup>12</sup>, inhibit mammary tumours in rats<sup>13</sup> and decrease platelet aggregation<sup>14</sup>. BR has been shown to inhibit methylcholanthrene induced sarcoma<sup>15</sup>, reduce myelosuppression induced by chemotherapy<sup>3</sup> and radiotherapy<sup>4</sup> and reduce lipid peroxidation in the mice liver<sup>4</sup>. Rasayanas contained extracts of several plants and some of them have been shown to possess immunomodulatory and antioxidant activity<sup>16,17</sup>. The activity of Rasayanas on immune system may be due to several compounds present in these preparation acting synergistically in the biological system.

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