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Leukocyte Adherence Inhibition (LAI) in Rats Bearing Transplantable Syngeneic Tumors of Different Immunogenicity

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Abstract—*The tube leukocyte adherence inhibition (LAI) assay was used to follow the LAI response in inbred BN rats with subcutaneously (s.c.) transplanted syngeneic liposarcoma (LS*175*) tumor and in inbred WAG rats with s.c. transplanted syngeneic colon (CC*531*) and skin* (1618) *tumors. Initially, the tube LAI assay was used to follow the LAI reactivity of peripheral blood leukocytes (PBL) from BN rats bearing tumor LS*175*. Sporadic LAI reactivity was observed in the PBL of these rats when incubated with a specific crude tumor extract for either* 2 *or* 20 hr. *No definite conclusion regarding the lack of significant tumor-specific LAI reactivity could be drawn since LS*175 *is a non-immunogenic tumor. Therefore additional LAI studies were performed with the weakly immunogenic tumor CC*531 *and the highly immunogenic tumor* 1618 *in WAG rats. Surprisingly, only sporadic LAI reactivity was observed in the PBL of rats bearing either the CC*531 *or the* 1618 *tumors using both the* 2 *and* 20 hr *LAI assays. This absence of tumor-specific LAI reactivity was particularly remarkable in the highly immunogenic* 1618 *tumor-bearing rats, since it would suggest that the antigen(s) responsible for its high immunogenicity could not evoke any consistent LAI reactivity. Since oxidative metabolites of arachidonic acid have been shown to be the final mediators of chemoattractant-induced LAI, the adherence inhibition effect of leukotriene B*4 *(LTB*4*) on the PBL of BN and WAG rats was also investigated in order to exclude the possibility that the lack of consistent LAI reactivity was due to a lack of responsive cell population in the PBL. PBL of both BN and WAG rats showed a significant increase in the adherence inhibition in the presence of LTB*4*, suggesting that the sporadic LAI reactivity was not due to a lack of responsive cell population.*

INTRODUCTION

THE LEUKOCYTE adherence inhibition (LAI) assay was first introduced by Halliday and Miller [1], and has been used successfully to monitor immune reactivity to a variety of antigens in man and experimental animals [2–5]. The LAI assay is based on the observation that sensitized leukocytes lose their ability to adhere to glass or plastic surfaces when exposed to sensitizing antigens *in vitro*. Since the introduction of the original hemocytometer LAI, several modified versions of the assay have been developed aiming at an automated, simple and dependable method. The tube LAI assay was originally developed to determine the specific sensitization of the organism to an immunizing antigen. This assay has been used with success to monitor cell-mediated immunity in cancer patients [6–8]. However, as reported earlier by us [9, 10], the proper selection of crude tumor antigen extracts is imperative for the successful execution of this assay in man. Extensive prescreening of the crude tumor extracts is an essential feature to exclude the false positive results. In addition, crude tumor extracts, which are initially highly discriminating in this assay, deteriorate on prolonged storage. This necessitates the regular replacement with new crude extracts. In order to overcome these two main drawbacks of the tube LAI assay, it became necessary to search for a well-defined animal tumor model which could be used to investigate and improve the conditions necessary for a more sensitive and reliable tube LAI assay.

Since the tube LAI assay has been successfully used to detect immunity in rats [11–15], and since three well-defined transplantable syngeneic tumors of increasing immunogenicity in rats were available, the animal studies reported here were undertaken.

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MATERIAL AND METHODS

Rats

Male rats of inbred WAG and BN strains were used. The animals were bred under specific-pathogen-free conditions and were 8–10 weeks old.

Tumors

Liposarcoma (LS175). LS175 is a liposarcoma which originated as a spontaneous tumor in a female BN rat in the pancreatic, retroperitoneal region. The tumor was observed when the animal was 80 weeks old [16]. Histopathological examination revealed that the infiltrating tumor was composed mainly of pale, swollen polyhedral cells with a granular cytoplasm containing fat. The tumor is palpable as early as 1 week after subcutaneous (s.c.) implantation. Immunization-challenge experiments, performed according to the method of Prehn and Main [17], revealed that LS175 is not immunogenic, i.e. the tumor growth is not inhibited or enhanced in immunized hosts. The tumor is transplantable in syngeneic animals.

Colon adenocarcinoma (CC531). CC531 was induced by treatment of WAG rats with 1,2-dimethylhydrazine (DMH). It originated in the ascending colon 40 weeks after six weekly injections of 30 mg/kg DMH [18]. The tumor is a moderately differentiated adenocarcinoma which is transplantable in syngeneic animals. When 2-mm cubes are implanted s.c., about 4 weeks are needed for the tumor to grow to a diameter of 10 mm. Classical immunization-challenge experiments [17] revealed that CC531 is weakly immunogenic, i.e. a slight but significant tumor growth inhibition is observed in immunized hosts.

Skin basal cell carcinoma (1618). 1618 is a transplantable radiation-induced basal cell carcinoma of the skin in the WAG rat [19]. The tumor has strong immunogenic properties, i.e. it does not grow in immunized hosts. Tumor 1618 has a doubling time of 2.5 days when implanted s.c.

Tumor implantation

Two-millimeter cubes of tumor LS175, CC531 or 1618 were implanted s.c. into the right flank of the experimental animals. LAI assays were performed about 14 days after implantation when the tumors were either palpable (tumors CC531 and 1618) or had reached a diameter of between 5 and 10 mm (tumor LS175). LAI assays were also performed sequentially in rats bearing tumor CC531 on day 2, 7, 14 and 21 after implantation.

Sensitization of WAG rats with irradiated 1618 tumor cells

1618 tumor cell suspensions were prepared from s.c. tumor implants. Tumor cells were isolated according to the method of Reinhold [20]. Cells were washed twice with RPMI 1640 medium (GIBCO), X-irradiated at a dose of 80 gy and mixed with Freund's incomplete adjuvant. WAG rats were immunized twice with a 14-day interval by intraperitoneal injections with 10^8 cells. LAI assays were performed 1 week after the first and 2 days after the second immunization.

Preparation of tissue extracts

Tumor tissue, obtained from s.c. implants, was resected aseptically and necrotic parts were removed. Tissue extracts were prepared as previously described [4]. Briefly, fatty and fibrous tissue was carefully dissected away and the specimen was cut into small pieces with scissors. A 20% (w/v) homogenate was prepared in ice-cold phosphate-buffered saline (PBS) using an Ultra-turrax TR 18–10 homogenizer. The homogenate was centrifuged at 1000 ***g*** for 5 min and subsequently at 50,000 ***g*** for another 60 min. The supernatant (crude tumor antigen extract) was sterilized by filtration through a 0.2 μm Millipore filter. In a later series of experiments the extracts were not filtered. Extracts of kidneys from normal WAG and BN rats were prepared in the same manner. The protein concentration of the stock extracts was determined using the Bio-Rad protein assay (Bio-Rad, Holland), and ranged from 3 to 7 mg/ml. All extracts were stored at −70°C in 1-ml aliquots and were used only once.

Isolation of peripheral blood leukocytes (PBL)

Peripheral blood from normal rats and rats with progressively growing tumor was drawn under ether anesthesia. Two to three milliliters of blood were collected from the tail vein into heparinized tubes. PBL were isolated by gradient centrifugation [21]. Briefly, blood was diluted 1:1 with RPMI 1640 medium, layered over lymphocyte separation medium (LSM, Litton Bionetics) and centrifuged for 15 min at 500 ***g***. Cells at the plasma/LSM interface were collected and washed twice in RPMI medium. After counting and viability testing using trypan blue dye exclusion, the suspension was adjusted to a concentration of 5×10^6 living cells/ml.

The LAI assay

The LAI assay was performed in triplicate in 12.5-ml round-bottomed glass tubes as described previously [4, 9, 10]. Each tube contained 0.3 ml RPMI medium, 0.1 ml of the leukocyte suspension (5×10^5 cells) and 0.1 ml (100 μg protein) of the tumor extract. The effect of Leukotriene B4 (LTB4) on BN and WAG leukocyte adherence was tested in a similar manner, with one set of tubes receiving different concentrations of LTB4 diluted in 0.1 ml of medium, and the other set receiving 0.1 ml medium alone. The tubes were placed in a near-horizontal position, so that the contents covered about 80% of the lower surface. They were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 2 or 20 hr the tubes

were placed in a vertical position and their contents mixed gently. A sample was withdrawn and the non-adherent cells were counted electronically in a Microcell counter (Toa Medical Electronics, Japan). The results were expressed as the non-adherent index (NAI), which was calculated using the formula:

$$\text{NAI} = \frac{A - B}{B} \times 100,$$

where A represents the number of non-adherent cells in the presence of specific antigen (experimental extract) and B stands for the number of non-adherent cells in the presence of non-specific antigen (control extract). The NAI values outside the limits of mean ± twice the standard deviation of the control group were considered to represent a significant effect.

RESULTS

When PBL from non-immunized controls or tumor-bearing rats were incubated in test tubes without the addition of tumor extract for either 2 or 20 hr, between 10 and 15% of the cells remained non-adherent. The addition of non-specific tumor extract resulted in an increase in the non-specific inhibition of adherence which was proportional to the protein concentration. Table 1 shows the representative results of a dose–effect study performed using the PBL of rats bearing CC531 tumor with 1618 tumor extract. Table 2 depicts the results of studies performed in CC531 tumor-bearers with CC531 tumor extracts. No significant increase in the non-adherence beyond a concentration of 100 μg was observed, indicating it to be an optimum concentration. Similar observations were made in LS175 tumor-bearers and 1618 tumor-bearers using LS175 and 1618 tumor extracts respectively.

Figure 1 shows the LAI results obtained in the initial studies with the non-immunogenic LS175 tumor-bearing BN rats. The LAI assay was performed using LS175 tumor extract as the specific antigen and extracts of normal kidney tissue and tumors CC531 and 1618 as non-specific antigens. The results were expressed as NAI. The NAI values outside the limits of mean ± 2 S.D. of the control group were considered to represent a significant effect. The results of the 2-hr LAI assay show that, using kidney extract or 1618 extract as non-specific antigens, none of the ten tumor-bearing animals showed any positive LAI reactivity (lanes 1 and 3 respectively). When CC531 extract was used as non-specific antigen, one tumor-bearer showed a positive LAI reactivity (lane 2). Increasing the LAI incubation time to 20 hr resulted in no significant improvement in the LAI reactivity. Using CC531 and 1618 extracts as non-specific antigens, only one animal in each group showed a positive LAI reactivity (lanes 2 and 3 respectively).

Table 1. Percentage of non-adherent PBL per LAI tube at various crude tumor antigen concentrations

Concentration of crude 1618 antigen extract (μg/tube)	Mean non-adherence (%) CC531 tumor-bearers (n = 6)	Normal rats (n = 6)
400	58 ± 9	56 ± 10
300	54 ± 8	55 ± 2
200	54 ± 8	57 ± 6
100	48 ± 11	53 ± 3
50	37 ± 13	42 ± 2
—	12 ± 4	14 ± 2

Table 2. Percentage of non-adherent PBL per LAI tube at various crude tumor antigen concentrations

Concentration of crude CC531 antigen extract (μg/tube)	Mean non-adherence (%) CC531 tumor-bearers (n = 10)	Normal rats (n = 10)
200	66 ± 7	59 ± 10
100	63 ± 8	54 ± 11
50	50 ± 10	40 ± 12
20	20 ± 5	17 ± 8
—	10 ± 7	12 ± 5

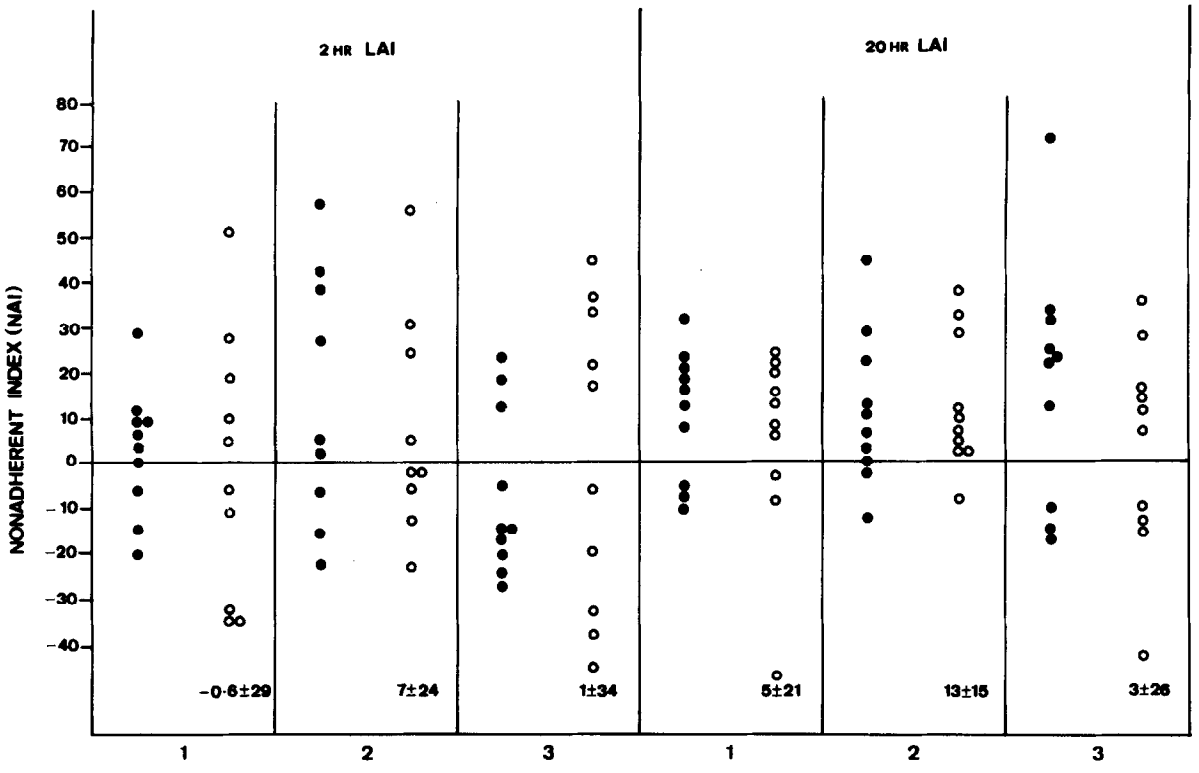

Fig. 1. Distribution of NAI values in tumor-bearing (●) and control BN rats (○). The LAI assays were performed 14 days *after s.c. implantation of tumor LS*175. *Extract of tumor LS*175 *served as specific antigen and extracts of normal BN kidneys* (1), *tumor CC*531 (2) *and tumor* 1618 (3) *as non-specific antigens. The mean ± S.D. of the NAI value of each control group is also shown. The NAI values outside the limits of mean ± 2 S.D. of the control group were considered to represent a significant effect.*

The results of the studies using the weakly immunogenic tumor CC531 are shown in Fig. 2. The LAI assays were performed using the same conditions as those described for LS175 tumor-bearing animals. Extract of CC531 was used as the specific antigen and extracts of normal kidneys, LS175 and 1618 as the non-specific antigens. The results show that positive LAI reactivity was observed in one out of ten tumor-bearers when kidney extract was used as non-specific antigen (lane 1). In the 20-hr LAI assay, none of the ten tumor-bearers showed any positive LAI reactivity. In a separate series of experiments in which WAG rats were sequentially monitored using the 2-hr LAI assay on days 2, 7, 14 and 21 after tumor implantation, positive LAI reactions were occasionally observed at day 14 in one or two animals from a group of ten. However, when tested on day 21, these animals did not show positive LAI reactivity.

Figure 3 shows the results obtained in WAG rats bearing the highly immunogenic 1618 tumor. In the 2-hr LAI assay, when 1618 extract was used as specific antigen and extracts of LS175 and CC531 as non-specific antigens, one out of ten tumor-bearers in each group showed a positive LAI reactivity (lanes 2 and 3 respectively). One tumor-bearer showed a positive LAI reactivity in the 20-hr LAI assay when 1618 extract was used as specific antigen and LS175 extract was used as non-specific antigen (lane 2). Similar results were also observed using both the LAI assays in WAG rats which had been immunized with 1618 tumor cell suspensions. None of the ten immunized animals showed any positive LAI reactivity either 1 week after the first immunization or 2 days after the second immunization.

The LAI response of PBL from normal BN and WAG rats to various doses of LTB4 showed that only 2×10^{-9} M LTB4 resulted in significant adherence inhibition. In the ten WAG rats the percentage non-adherence ranged from 14 to 29 (mean 21 ± 6) in the presence of 2×10^{-9} M LTB4, whereas they ranged from 10 to 15 (mean 11 ± 2) in the absence of LTB4. Similar results were observed in BN rats.

DISCUSSION

Since its original development and introduction, the modified tube LAI assay has been used by several authors to detect cell-mediated antitumor immunity in rats [11–13, 15].

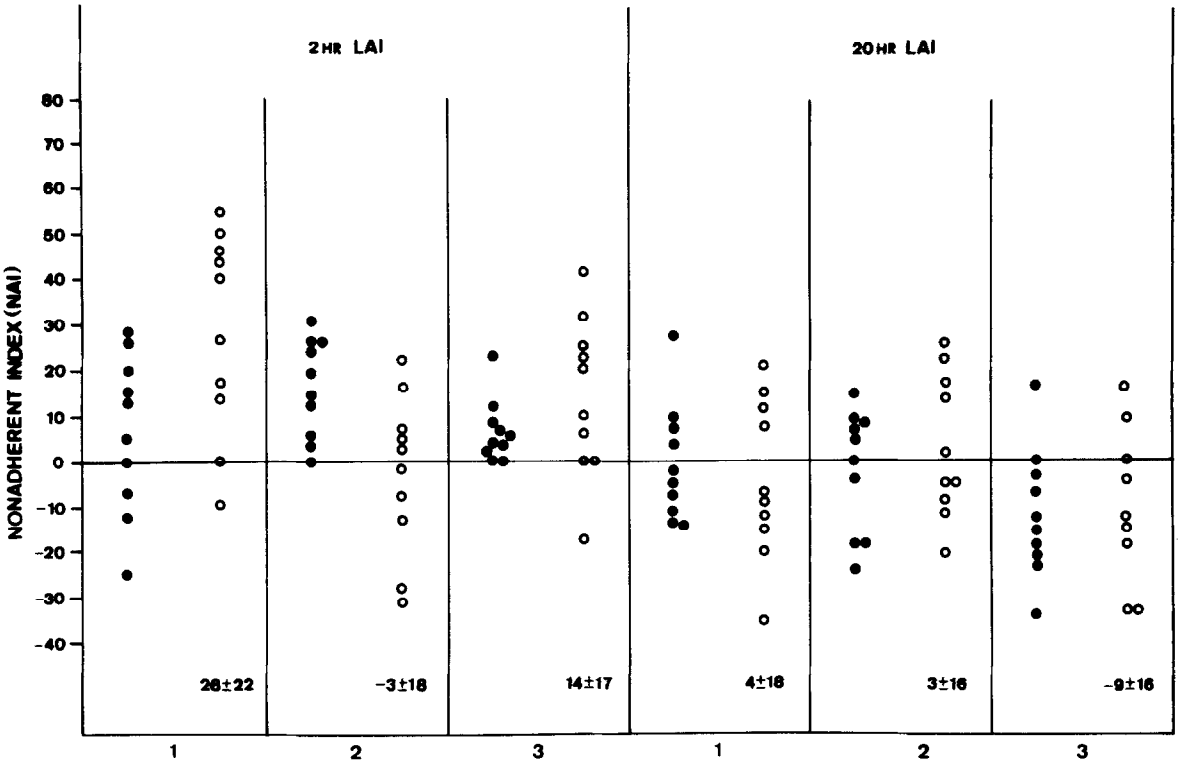

Fig. 2. Distribution of NAI values in tumor-bearing (●) and control WAG rats (○). The LAI assays were performed 14 days *after s.c. implantation of tumor CC*531. *Extract of tumor CC*531 *served as specific antigen and extracts of normal WAG kidneys* (1), *tumor LS*175(2) *and tumor* 1618 (3) *as non-specific antigens. The mean ± S.D. of the NAI value of each control group is also shown. The NAI values outside the limits of mean ± 2 S.D. of the control group were considered to represent a significant effect.*

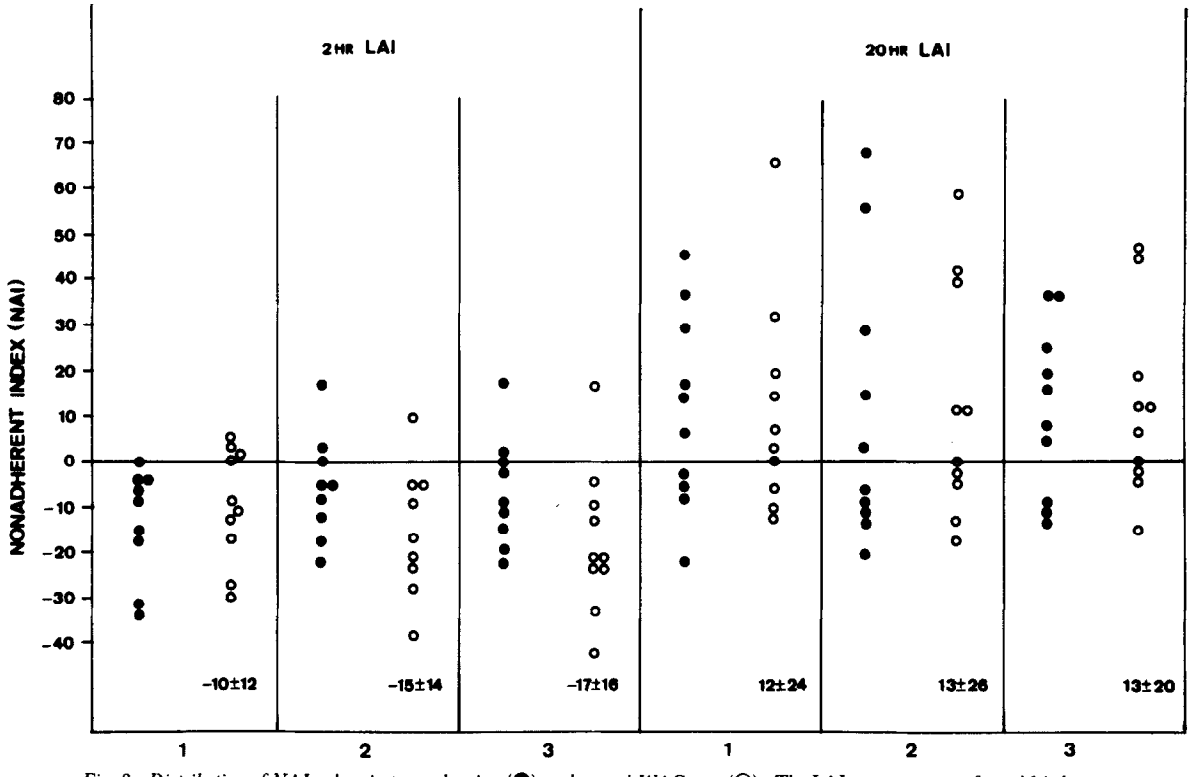

Fig. 3. Distribution of NAI values in tumor-bearing (●) and control WAG rats (○). The LAI assays were performed 14 days *after s.c. implantation of tumor* 1618. *Extract of tumor* 1618 *served as specific antigen and extracts of normal WAG kidneys* (1), *tumor LS*175 (2) *and tumor CC*531 (3) *as non-specific antigens. The mean ± S.D. of the NAI value of each control group is also shown. The NAI values outside the limits of mean ± 2 S.D. of the control group were considered to represent a significant effect.*

In the present studies the 2-hr tube LAI assay which was successfully used in humans [9, 10] has been used to follow the LAI reactivity of the peripheral blood leukocytes (PBL) of rats bearing transplantable syngeneic tumors. The initial LAI studies were performed in BN rats bearing a transplantable syngeneic LS175 tumor. The results obtained show that using the conventional 2-hr tube LAI assay, sporadic tumor-specific LAI reactivity was observed. In previous studies in rats bearing syngeneic tumors, Kalafut *et al.* [12] and Hung *et al.* [13] observed that prolongation of the LAI assay incubation time to 20-hr gave better and more consistent results than the 2-hr conventional assay. It was therefore decided to use in addition the 20-hr tube LAI assay. Once again, only sporadic tumor-specific LAI reactivity was observed in the PBL of tumor-bearers. There was no qualitative difference between the results of the two LAI assays used. The possible explanation for this lack of significant tumor-specific LAI reactivity could be that the LS175 tumor, which is non-immunogenic, does not or only weakly evokes an immune response which is occasionally detected in the tube LAI assay.

To investigate this possibility, LAI studies were performed using PBL of WAG rats bearing a weakly immunogenic CC531 tumor. Sporadic tumor-specific LAI reactivity was observed using both the LAI assays. Occasional positive LAI reactions were noted when CC531-implanted rats were sequentially monitored on days 2, 7, 14 and 21. The results obtained using the highly immunogenic tumor 1618 were similar to those obtained with the non-immunogenic and the weakly immunogenic tumors. The results of studies using WAG rats immunized with cell suspensions of tumor 1618 also failed to demonstrate a consistent tumor-specific LAI reactivity (results not shown).

The results presented in this paper contradict those obtained earlier by Kalafut *et al.* [12] and Hung *et al.* [13] and those reported recently by Morizane and Sjögren [14, 15]. All of these authors were able to demonstrate significant tumor-specific LAI reactivity in PBL of tumor-bearing rats. In the studies by Kalafut *et al.* and Hung *et al.* the 20-hr LAI assay was performed using homologous serum and fetal calf serum (FCS)-supplemented media, respectively. FCS-supplemented medium was also used in the micro-glass-tube LAI assay by Morizane and Sjögren. In the current studies, when the tube LAI assays were performed using FCS-supplemented medium, no tumor-specific LAI reactions were observed with the PBL of the rats bearing LS175, CC531 and 1618 tumors. An FCS concentration of 1% in the assay system totally inhibited the adherence of PBL from both normal and tumor-bearing rats. Similar observations were reported previously [11, 22, 23]. In those studies, normal serum or FCS inhibited the adherence of normal and 'immune' leukocytes and abolished the specific LAI reactions in the tube LAI assay.

The failure to observe any significant tumor-specific LAI reactions in this study could be due to the following reasons. Firstly, a lack of cells in the PBL that mediate the specific LAI reaction. It has been shown previously that in the tube LAI assay, cells of the monocyte/macrophage series play a central role in triggering the cascade of events leading to non-adherence [24]. Peritoneal cell suspensions (PC) contain mainly cells of the monocyte/macrophage series. In previous studies Holan *et al.* [3] observed specific LAI reactions in the PC of rats immunized with various antigens. However, when we performed pilot studies with the PC of LS175, CC531 and 1618 tumor-bearing rats, no tumor-specific LAI reactions were observed. Since oxidative metabolites of arachidonic acid are the final mediators of chemoattractant-induced LAI [25], the adherence inhibition effect of LTB4 on the PBL of BN and WAG rats was also investigated in order to exclude the possibility that the lack of consistent LAI reactivity was due to a lack of responsive cell population in the PBL. In both BN and WAG rats, 2×10^{-9} M of LTB4 significantly (two-fold) inhibited the adherence of PBL. This excludes the lack of responsive cells which could account for the sporadic LAI reactivity.

The sporadic tumor-specific LAI reactivity in this study could also have been due to an insufficient amount of LAI-reactive tumor antigen in the crude tumor extracts since all the extracts had been filtered. However, in a later series of experiments when unfiltered tumor extracts were used, sporadic tumor-specific LAI reactivity was observed in all three types of tumor-bearers. Purification of the crude extracts could possibly lead to a higher number of specific reactions. However, since sporadic LAI responses were observed in all three types of tumor-bearers, the possibility of obtaining significant tumor-specific LAI responses using purified extracts has to be considered rather remote.

Finally, it is possible that the site of tumor implantation in the current studies was inappropriate to evoke a specific LAI response. However, it is strange that the same tumor implantation site in the immunization-challenge experiments resulted in the tumor-specific immunity. The unlikely possibility remains that PBL are poor effectors of tumor-associated immunity of s.c.-implanted tumors. Perhaps the leukocytes isolated from regional draining lymph nodes would be more suitable as tube LAI effector cells. Transplantable syngeneic tumors may also induce suppressor cells or factors

which impair the immune response of the host [26]. Therefore, it might be assumed that consistent tumor-specific LAI reactions can be successfully detected only in animals with a primary autochthonous tumor.

In conclusion, the fact remains that tumor-specific LAI reactivity in rats is puzzling and may be even non-existent. Our initial goal to use the rat model in parallel studies for the improvement of the human tube LAI assay can be considered not to be feasible as yet.

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