PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **180**, 224–230 (1985)

Pulmonary Compartmentalization of Interferon and Natural Killer Cell Activity (42168)

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Abstract. Studies were performed to determine if natural killer (NK) activity in the mononuclear cells harvested from infected lungs was dependent on local or systemic factors. Mice were inoculated by intratracheal (it), intraperitoneal (ip), or intravenous (iv) routes with (a LD_{50} dose of) influenza virus A PR/8/34. At various days postinoculation cells from lungs, spleens, and peripheral blood were assayed for NK activity, and lung wash, lung homogenates, and serum were assayed for interferon. After it inoculation there was three- to fourfold increase of NK activity in the lung with little or no increase in NK activity in spleens or peripheral blood. The local augmentation of NK activity in the lung correlated with an increase in interferon (IFN) titer in the lung wash and lung homogenate of PR8 inoculated mice. The virus failed to induce IFN or augment NK activity when it was inoculated systemically. The observed local augmentation of NK activity and local induction of interferon production following it inoculation suggests that the NK population in the lung is capable of responding to locally derived regulatory factors.

In the last several years, the existence of a naturally occurring cytolytic cell population capable of lysing various tumor targets and virally infected targets without previous immunologic sensitization has been described (1, 2). These cells, termed natural killer cells (NK), have been detected in cells harvested from peripheral blood, bone marrow, spleen, lymph nodes, gastrointestinal tract, and lung (3–7). It has been suggested that these cells may play a role as a first line of defense against virus infection by mediating lysis of virus infected cells prior to the development of specific humoral and cell-mediated defense mechanisms (8–11). In support of this hypothesis is the evidence that interferon and interferon inducers (e.g., virus, poly(I):(C), LPS) have been shown to augment NK activity both *in vitro* and *in vivo* (12–15).

We have previously shown that the lung is a source of NK activity and that cells mediating NK activity in the lung demonstrate cell surface antigens and target preference which are similar to those described for NK cells harvested from spleens (4). It is not clear if this NK activity is regulated locally in the lung or is dependent on systemic regulation. At that time we reported that following local inoculation of a larger dose of influenza virus (125 HAU) there was an augmentation of NK activity in cells harvested from the lung homogenates 2 days postinoculation that was not accompanied by augmentation of NK activity in cells obtained from the spleen until Day 4 (4). (This dose was 100% lethal for all mice by Day 10.) Previous reports indicate that influenza virus infection in the lung induces interferon production in the lung (16); based on those observations it is possible that NK activity in the lung was augmented by interferon that was produced locally in response to the influenza virus infection. The inability of a pulmonary virus infection to augment NK activity systemically (in the spleen) may have been due to an inadequate amount of (systemic) interferon that could be "sensed" by NK cells in the spleen. To test this hypothesis we designed studies to determine if inoculation of a LD_{50} dose of infectious influenza virus by various routes could induce the production of interferon and/or augmentation of NK activity in the lung or systemic tissues. The data in this paper demonstrate that intratracheal (it) inoculation of mice resulted in a rise in pulmonary interferon titer that correlated with increase in NK activity in lungs but not in

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0037-9727/85 $1.50

spleen or peripheral blood. It was also observed that intravenous (iv) or intraperitoneal (ip) inoculation of the LD_{50} dose of virus was a poor inducer of interferon and produced little or no augmentation of NK activity systemically or in the lung.

Materials and Methods. *Mice.* F_1 mice (C57BL/6 × DBA/2) were purchased from the Jackson Laboratory (Bar Harbor, Maine). The experimental mice were 9 to 12 weeks of age, and all experiments were done with age and sex matched animals.

Preparation of lymphoid cells for NK. Suspensions were made from lung and spleen tissue. Mice were killed by cervical dislocation and lungs were removed, minced finely, and incubated with 120 units of collagenase (Sigma Chemical, St. Louis, Mo.) in 15 ml media per every two lungs for 90 min. To dissociate into single cell suspensions, minced spleens or collagenase-treated lungs were gently tapped through a stainless-steel screen after the method of Billingham and Silvers (17). Cells were washed, counted by phase microscopy using trypan blue dye exclusion to identify viable cells, and resuspended to appropriate concentrations. Peripheral blood was obtained from retroorbital plexus 15 min after intraperitoneal injection of 50 units of heparin. Blood was diluted 1:4 with phosphate buffered saline and carefully layered over 70% percol and centrifuged 30 min at 200*g*. Mononuclear cells harvested from the interface were counted as above and resuspended to appropriate concentrations.

Preparation of fluids for interferon titers. Lung wash, lung homogenate, and serum were harvested from groups of three animals at each time point and assayed for interferon. Lung washes were standardized by injecting 1.5 ml sterile phosphate buffered saline into each lung via catheter and withdrawing as much as possible.

Recoveries ranged from 0.8 to 1.2 ml. Washes were clarified to remove cells and debris by centrifugation for 10 min at 2500 rpm and filtered through 0.45-μm Millipore filter. Washed lungs were removed from the animal and homogenized in a tissue homogenizer and resuspended to a total volume of 2 ml with phosphate buffered saline. Homogenates were clarified by centrifugation, as for washes, and filtered through 0.45-μm Millipore filters. Serum was obtained by allowing blood to clot, then centrifuging to separate cells from serum. All specimens were frozen (−70°C) until assayed for interferon.

Tumor cells. YAC-1 (H-2^a, lymphoma) were maintained *in vitro* in RPMI 1640 as described (18).

NK cell assay. Approximately 5×10^6 tumor cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Searle, Arlington Heights, Ill.) in 0.5 ml of RPMI 1640 medium containing 5% FBS for 1.5 hr. The cells were washed three times and diluted to a final concentration of 2×10^5 tumor cells/ml. Targets (2×10^4 in 0.1 ml of complete RPMI 1640 medium) were placed in wells of round-bottomed 96-well plastic microtiter plates (Costar, Cambridge, Mass.) with varying numbers of fresh spleen cells, lung cells, or peripheral blood cells, in 0.1 ml of complete RPMI medium. Generally each cell suspension was plated at three different effector to target cell (E:T) ratios ranging from 50:1 to 12.5:1. Cells in each ratio were plated in quadruplicate. The incubation periods were 4 hr for YAC-1. After incubation the plates were centrifuged at 200*g*, and 0.1 ml of supernatant was harvested from each well and counted in a Packard Prias scintillation counter (Packard Instrument Co. Inc., Downers Grove, Ill.). The mean percentage specific cytotoxicity was calculated as

$$\text{mean \% cytotoxicity} = \text{cpm}\frac{\text{experimental} - \text{spontaneous (med)}}{\text{maximal } (H_2O) - \text{spontaneous (med)}} \times 100.$$

The variation between the replicates was less than 5%. Spontaneous release values did not exceed 20% of the maximum release value in the experiments reported herein. Experiments were repeated a minimum of three times. Representative experiments are reported under Results.

Cell pretreatment. Macrophages were removed from lung mononuclear cell populations (2×10^6 per ml RPMI 1640, 10% FBS) by incubation at 37°C, 5% CO_2 for 2 hr on plastic dishes (Corning, N.Y.). Nonadherent cells were removed by washing with medium. For treatment with anti-asialo GM1 (Wako

Chemicals, Dallas, Tex.) 20×10^6 macrophage depleted cells in 1 ml RPMI 1640 were first incubated at 4°C with 1:200 dilution of antisera followed by 1:8 dilution of rabbit C.

Assay for IFN. The antiviral titer of the putative interferon samples was determined by plaque reduction of the Indiana strain of vesicular stomatitis virus or murine L-929 cells. The antiviral titer corresponded to the reciprocal of the greatest dilution of test sample that reduced virus plaques by 50%. One interferon unit equaled 0.88 NIH G-002-904-511 reference units in this assay.

The interferon was characterized by treatment at pH 2 for 1 hr, and treatment with anti-interferon antibodies as previously described (19). Anti-IFN-α/β was obtained from Lee Biomolecular, San Diego, California. Anti-IFN-γ was obtained from Enzo Biochemical New York, New York.

Influenza virus (20). Virus (PR/8/34, H1N1) was a sucrose gradient-purified virus concentrate (a generous gift from M. Phelan, Ph.D. of the Virology Department, Bureau of Biologics). Influenza virus (0.125 hemagglutinating units (HAU) in 50 μl of PBS) was inoculated into mice (anesthetized with chloral hydrate) through a 23-gauge needle inserted into a catheter within a cannula placed in the trachea via the mouth. The (LD_{50}) dose of virus used caused 50% of the mice to die from infection by Day 8, the remaining 50% survived.

Results. *Pulmonary influenza virus infection augments NK activity in the lung but not the spleen.* B6D2F1 mice (9–12 weeks old) were inoculated as a group with 0.125 HAU influenza virus A PR/8/34 directly into the trachea. At 2, 4, 6, 8, and 10 days postinoculation groups of four mice were killed and lung, spleen, and peripheral blood cells were prepared and assayed for NK activity as described under Materials and Methods. By Day 4 postinoculation natural killer activity in cells harvested from the lungs of infected mice (Fig. 1) was greater than 300% of that seen in uninoculated control animals and by Day 6 postinoculation peaked at greater than 400% of controls and by Day 8 the level of augmentation began to drop. The cytotoxicity of YAC-1 cells observed on Days 4 and 6 postvirus inoculation was associated with the mononuclear cells that did not adhere to plastic during a 2-hr incubation at 37°C, 5% CO_2; the ability to lyse YAC-1 target cells could be eliminated by prior incubation with a rabbit antiserum to asialo GM1 and complement (Table I). From these data we concluded that the assays were testing NK activity. The NK activity in spleen and peripheral blood cells from the same animals which was only slightly elevated above normal values on this day did not rise significantly throughout the test period. These data demonstrate that during a pulmonary infection with influenza virus (at a LD_{50} dose) there was a three- to fourfold augmentation of NK activity locally in the lung without a similar augmentation in systemic tissues, represented by spleen and peripheral blood cells.

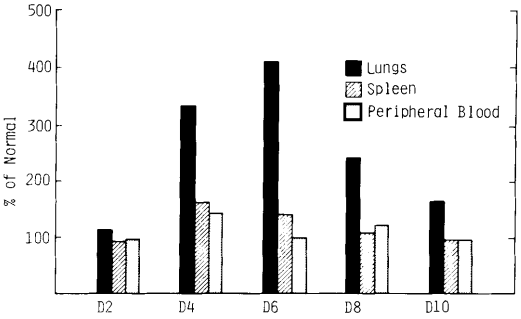

FIG. 1. Detection of natural killer cell activity in lung, spleen, and peripheral blood of influenza infected mice. B6D2F1 mice were inoculated with 0.125 HAU of influenza virus A PR/8/34 by the intratracheal route. On days indicated on abscissa, cells from lung, spleen, and peripheral blood of four animals were pooled and assayed for natural killer activity (YAC-1) in a 4-hr chromium release assay. The cells were plated at 50:1, 25:1, and 12.5:1 effector:target (E:T) ratio. Results from 25:1 E:T ratio are illustrated on histogram. The lysis by cells from infected animals was compared to lysis by cells from uninoculated animals and expressed as percentage of normal. Normal values ranged from 10 to 15% NK lysis. % normal = NK lysis infected/NK lysis (normal) × 100.

Systemic inoculation of virus does not augment NK in the lung or the spleen. As with the intratracheal inoculation with virus (9–12), groups of mice B6D2F1 were inoculated with 0.125 HAU influenza virus A PR/8/34 either by intraperitoneal or intravenous routes. At 2, 4, 6, 8, and 10 days postinoculation mice were killed and lung, spleen, and peripheral blood cells were prepared and assayed for NK activity as described under Materials and Methods. Systemic (iv and ip) inoculation of influenza virus did not result in augmentation of NK activity in the lung or systemic tissues

TABLE I. CHARACTERIZATION OF PULMONARY CELLS THAT LYSE YAC-1 TARGET CELLS

Animal[a] pretreatment	Treatment[b] of lung cells	% ^{51}Chromium release (SEM < 5%) Days post IT inoculation (E:T—50:1) 4	6
it, PR/8/34	None	26.1	30.6
	Nonadherent + C	31.2	30.6
	Nonadherent + R AGM1 + C	3.9	4.5
it, PBS	None	11.3	9.5
	Nonadherent + C	8.7	10.5
	Nonadherent + R AGM1 + C	1.3	1.4

[a] B6D2F1 mice were inoculated intratracheally with 50 μl of 0.125 HAU of influenza virus PR/8/34 or with 50 μl of PBS. Four and six days later mononuclear cells were harvested from collagenase-treated lung tissue and tested for their ability to lyse ^{51}Cr-labeled YAC-1 target cells.

[b] Mononuclear cells from harvested lungs were tested either directly or after removal of macrophages nonadherent to plastic after 2 hr, 37°C, 5% CO_2, and NK cells by subsequent incubation with rabbit anti-asialo GM1 (R AGM1 and complement C).

(spleen and peripheral blood cells). Following intraperitoneal inoculation the NK activity of cells in lungs ranged from 56% of the activity of cells harvested from uninoculated animals (normal) on Day 2 to a maximum of 159% of normals on Day 4 (Table II). Similarly NK activity detected in cells from spleen of ip inoculated animals varied little from peripheral blood NK activity detected in the respective tissues from normal uninoculated mice. Following intravenous inoculation, there was little difference in NK activity detected in mononuclear cells harvested from inoculated animals versus from cells harvested from the uninoculated control animals. The possibility is raised that systemic inoculation of influenza virus does not augment NK activity because there is a lack of systemic factors (interferon) that can augment the NK activity. This possibility was investigated by first assaying for a rise in interferon in the tissues (lung) where NK activity was augmented.

Interferon titers rise in lung wash and lung homogenates but not serum of mice inoculated intratracheally but not systemically with influenza virus. Mice were inoculated with 0.125 HAU of influenza virus A PR/8/34 by intratracheal, intraperitoneal, or intravenous routes. At various days postinoculation groups of three mice were killed and lung wash, lung homogenates, and serum were assayed for interferon as described under Materials and Methods. Interferon titers began to rise substantially in lung wash and lung homogenate of intratracheally inoculated mice by Day 4 reaching titers of 365 and 343, respectively (Fig. 2). At Day 6 postinoculation the interferon titer in lung wash of it inoculated mice peaked at 388 and remained high in lung homogenates with a titer of 200. By Day 8 the interferon titers were beginning to drop with a lung wash titer of 150 and a lung homogenate titer of 33. By Day 10 the interferon titers in lung wash and lung homogenates had dropped to levels of uninoculated mice. The serum of intratracheally inoculated mice did not contain detectable levels of interferon except for a slight increase in titer at Day 4. However, the increase in serum interferon did not correlate with a similar increase in NK activity in the peripheral blood nor did mice inoculated with virus by intraperitoneal or intravenous routes, at same dose used for the intratracheal inoculations, produce detectable levels of interferon (data not shown). These results demonstrate that influenza virus infection of the lung can result in increased interferon production locally (lung wash and lung homogenates) without a similar increase in interferon titers in the serum. Intravenous or intraperitoneal inoculation of a dose of virus equivalent to that used in intratracheal inoculations did not induce the production of interferon that could be detected in serum or in the fluids harvested from the lung.

The interferon produced by mice inoculated with virus by the intratracheal route was characterized as primarily interferon-α/β. This was due to the relative pH 2 resistance, 56°C sensitivity, sensitivity to an anti-IFN-α/β serum, and resistance to an anti-IFN-γ serum (21) (see Materials and Methods).

Discussion. The results of these studies demonstrate that during pulmonary influenza virus infection initiated by a LD_{50} dose of PR/8/34 given intratracheally, natural killer activity was augmented locally in the lung without a concomitant augmentation of NK activity in systemic tissues (spleen and peripheral blood). The local (lung) augmentation of NK

TABLE II. NATURAL KILLER CELL ACTIVITY OF SYSTEMICALLY INOCULATED MICE[a]

Route of inoculation	Tissue tested	Exp. No.	% Normal[b] D2	D4	D6	D8	D10
Intravenous (iv)	Lung	1	89	138	107	68	104
		2	92	114	219	49	119
	Spleen	1	90	109	131	86	71
		2	99	126	123	82	92
	PB	1	98	85	105	95	98
		2	155	133	80	118	114
Intraperitoneal (ip)	Lung	3	64	159	97	85	125
		4	56	90	136	93	128
	Spleen	3	114	80	127	98	120
		4	102	107	68	106	127
	PB	3	113	117	71	115	88
		4	126	63	100	180	68

[a] B6D2F1 mice (9–12 weeks old) were inoculated with 0.125 HAU of PR/8/34 by either iv or ip inoculation. On days indicated mononuclear cells from lung, spleen, and peripheral blood (PB) of four animals were pooled and assayed for natural killer activity (YAC-1) in a 4-hr chromium release assay. The cells were plated at 50:1, 25:1, and 12.5:1 effector:target (E:T) ratio. Results from 25:1 E:T are shown.

[b] The lysis by cells from infected animals is compared to lysis by cells from uninoculated animals and expressed as percentage of normal. Normal values varied from 10–15% NK lysis. % normal = NK lysis (infected)/NK lysis (normal) × 100.

activity correlated with a rise in local (lung) interferon titers. The interferon that was produced in the lungs of intratracheally inoculated mice was interferon-α/β (19, 22). When the same dose of virus was inoculated by systemic routes (intravenous or intraperitoneal) little or no increase in NK activity or interferon was detected in systemic tissue or in the lung. Previous studies in our laboratory have demonstrated that a higher dose of influenza virus could augment NK activity in the spleen 2 days after the pulmonary augmentation is observed, thus this finding is not absolute but relative to the dose used. Using the LD_{50} dose of influenza it is possible to dissect the factors that may regulate pulmonary defenses to influenza virus infection in the absence of systemic events. Furthermore, under the experimental conditions described a respiratory virus infection can induce regulatory factors and effector cells locally within the lung when and where they may be needed for pulmonary defense. Also, it may be concluded from the above data that *local* populations of NK cells in the lung are the cells responding to interferon production in the lung during pulmonary influenza virus infection.

Previous reports have shown augmented NK response to virus using Kunjin (23), Sindbis virus (24), murine cytomegalovirus (25), and Lymphocytic Choriomeningitis virus (26). Leung and Ada (27) also reported a response of augmented NK activity in lungs of mice inoculated by a sublethal dose of influenza virus. In contrast to our studies they, however, observed that intravenous inoculation of virus

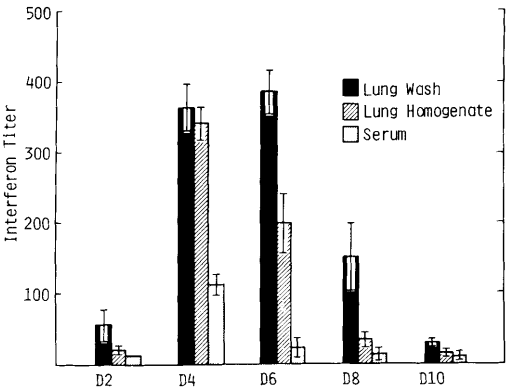

FIG. 2. Detection of interferon in lung wash, lung homogenate, and serum of intratracheally inoculated mice. B6D2F1 mice were inoculated with 0.125 HAU of influenza virus A PR/8/34 by the intratracheal route. On days indicated on abscissa lung wash, lung homogenate, and serum were harvested from groups of three animals and assayed for interferon titer by a standard VSV plaque reduction assay. The interferon titer is the reciprocal of the highest dilution giving a 50% reduction of plaques.

resulted in augmentation of NK activity in the spleen. This difference in observations may be due to differences in the number of HAU of influenza virus inoculated. It has not been demonstrated previously that the response to influenza virus may be generated locally by locally derived regulatory factors acting on a local population of effector cells.

The observation that pulmonary influenza infection can induce increases in NK activity locally without an increase in NK activity in major systemic tissues that are known as reservoirs for NK cells raises the possibility that NK cells exist locally within the lung. Therefore, an important issue is raised by these studies. Where are the NK cells within the lung located? The NK cells could be either in the pulmonary capillaries or within the pulmonary interstitium within an extravascular compartment. One would expect that NK cells in the interstitium would be responsive to local factors, however, if the NK cells are within the capillaries then the observation that the augmented activity can only be detected locally raises the possibility that once NK cells are "activated" they do not recirculate. Such augmented NK cells may perhaps be better able to migrate into the pulmonary interstitium therefore giving the impression of an extravascular source of NK cells in the lung. Confining augmentation of NK activity to the local environment at the site of the infection may prevent untoward lytic activity by activated NK cells in uninfected tissues (28).

It is possible that the natural killer activity that is augmented early during a pulmonary virus infection, prior to the development of specific humoral and cellular responses by the host, may aid interferon and other host defenses in controlling virus replication in the initial stages of infection. During the preparation of this manuscript we have completed other studies that demonstrate the survival time of the mice to influenza virus infection with the LD_{50} dose can be decreased by selectively depleting the NK activity within the lung by intratracheal administration of an antibody to asialo GM1, a neutral glycospingolipid expressed on NK cells (manuscript submitted for publication). Thus, a hypothesis can be formulated that states the local defenses within the lung are of primary importance in protection and recovery from respiratory infections. Therefore the possibility is raised that individuals who may not produce an adequate cellular (T lymphocyte) immune response to influenza virus may be aided more effectively by *local* treatment of the lung than by systemic inoculation with interferon or interferon inducers to augment their NK activity.

The authors thank Ms. Judy Guffee for her expert technical assistance, and Ms. Pattie Pipes and Aquillia Rolle for preparation of the manuscript. We also appreciate the many helpful discussions concerning these studies with Dr. Michael Bennett and Dr. Vinay Kumar.

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Received March 6, 1985. P.S.E.B.M. 1985, Vol. 180.
Accepted May 20, 1985.