# CHAPTER V

## DISCUSSION

Successful suppression and eradication of rabies infection requires a rapid immunological responses of cellular and humoral arms. Although effective neutralizing antibody has been considered responsible for abortive infection in mice that are genetically resistant to rabies virus infection (61,62), this work did not study the significance of killer cell system. Nevertheless, no correlation was found between the appearance of antibody and survival in mice (6), although it was necessary for protection in vaccine efficacy trial in dogs (188). Lack of cytotoxic killing function, as examined by using spleen cells as effectors, parallel the degree of mortality in mice (7). Cells with killing function can be classified into a fast-responding group called natural killer cells. These require no MHC restriction, previous contact, or cytotoxic T and K cells. Cytotoxic T cells bind target while recognizing antigen and MHC determinant. K cells recognize the Fc of IgG antibody bound to antigen on the target cell surface.

In presymptomatic rabies the initial site where immune cells may come to contact with virus antigen may be the inoculation area or the dorsal root ganglion where the blood nerve barrier is scant. An interaction between the antigen and cells at the inoculation site may not be effective since the incubation period in these patients is prolonged and in some circumstances this might be as late as 6 years (27). Due to early appearance of rabies neutralizing antibody, occasionally within 3-5 days after the first prodrome, the most likely site of immune recognition could be the nerve ganglion (1,88). However, not more than 20% of symtomatic rabies patients can develop an adequate antibody response. This may be due to defective immune recognition of the nucleocapsid rabies antigen. It is still not known what mechanism and which step of immune process is responsible for this defect. The error may start early at the stage of antigen processing, or it may involve interaction between macrohage and T cells, clonal proliferation and differentiation and maturation of T and B cells. Theoretically, this may also influence the development of specific cytotoxic T cells. N protein is not only crucial for elicitation of neutralizing antibody, it has been shown to protect animals having received pure N vaccine from rabies virus challenge. These animals survive without developing detectable neutralizing antibody thus implicating the role of cellular immunity. Once the virus reaches the dorsal root ganglion, it gains access and then spreads rapidly throughout the central nervous system.

Following the onset of neurological symptoms, death occurs usually within 7 days. Therefore, due to a rapidly progressive course of the disease and defective N recognition impairing antibody development and possibly specific cellular reaction, fast responding NK killer cells are a major protective mechanism in rabies. Any failure of NK in terms of number and function may explain virulence of rabies in man. NK has been shown to be important in herpes infection. Individuals lacking of only NK function had recurrent disseminated herpes infection (15). NK numbers, as determined by monoclonal antibody to Leu 7 (CD 57), have been reported to be diminished in 7 rabies patients regardless of clinical types (16). However, NK function in rabies patients has never been determined . Furthermore, CD 57 has been shown to be common in a variable portion of CD 3+ 8+ lymphocytes which have low or no spontaneous cytotoxicity (107). Hercend et al (96) and Lanier et al (109) have described NKH1 or Leu 19 or CD 56 monoclonal antibody that specifically react to more than 95 % of NK in peripheral blood.

Our results revealed that the number of CD 56 cells was comparable in rabies patients and normal controls. Two out of 6 non-rabies encephalitic patients had a diminished number of circulating CD 56 cells yet all recovered. The number of circulating NK cells thus may not predict the clinical outcome since these cells are able to proliferate after activation and it is possible that these cells are sequestrated at the site of infection and help clearing infective agents from the CNS. This

phenomenon has also been demonstrated in acute Sindbis virus encephalitis in mice (12). In this study, although NK number was not determined, level of NK activity was preferentially increased in the CSF of the infected mice as compared to that in the peripheral blood. Thus, the absence of diminution of circulating NK numbers in rabies patients may indicate lack of an adequate defense response. Our rabies patients had only slight CSF pleocytosis thus not adequate for NK study.

The use of the CD 57 monoclonal antibody (HNK-1 antibody) was not specific for NK cells since previous studies had clearly established that this antibody (a) reacted only with a variable proportion of cytotoxic NK cells (from 20 to 60%)(100), (b) did not react with presumably immature NK cells in human cord blood (99), and (c) reacted with a significant proportion of cells with the surface phenotype of mature T cells, either OKT8 or OKT4 positive (107), suggesting that the antigen detected may be present at some stage of differentiation and/or activation of T cells and of other hematopoietic and nonhematopoietic cell types.

We also studied the number of CD 57 cells in rabies, non-rabies patients and controls. CD 57 cells were significantly decreased in both rabies and non-rabies groups ($p<0.05$) as has been previously described (16). The reduction of CD 57+ cells in rabies patients may be due to the simultaneous reduction of other populations od CD 57+ cells besides NK cells. These populations of CD 57+ non-NK cells may be CD3+8+ lymphocytes possessing no or

low NK activity (107). Furthermore, circulating CD 8 in rabies were depressed while CD 8 but not CD 57 cells could be demonstrated in the brains of 2 rabies patients using immunohistochemical technique (S.Tirawatanapong, unpublished results). The cause of this CD 57 depression needs further study.

NK function in rabies patients and controls were comparable as determined by 4-hr microcytotoxicity assay. However, it is important to note that their response could still be further enhanced in vitro whereas this enhancement was not demonstrable in patients with non-rabies encephalitis who survived. Submaximal function and possibly lack of sequestration of NK cells in the CNS may, therefore, in addition to the other defects be responsible for rabies virulence in man.

Conclusions :

1. The number of NK cells of CD 56 phenotype in the rabies group was comparable to that in controls.
2. Diminished cells of CD 57 phenotype were found in all groups of encephalitic patients regardless of the type of etiologic organisms.
3. It is intriguing to speculate what is responsible for the depressed level of cells with CD 57 phenotype.
4. 4-hr Microcytotoxicity assay of NK function revealed comparable results between controls and rabies group.

5. In vitro enhancement of NK cells activity with $\alpha$-IFN or IL-2 was observed only in a group of rabies patients, this phenomenon could not be demonstrated in non-rabies encephalitic group.

6. Incremented difference between pre and post stimulation with IL-2 was greater in the control group than that in the rabies group but with $\alpha$-IFN the result was comparable.

7. A similar, but not identical response of NK cells, in terms of number and function which was observed in rabies patients may be interpreted as being a failure to stimulate or activate adequately these fast responding barrier.

8. According to our previous studies (submitted for publication) on the levels of soluble interleukin-2 receptor (sIL-2r) and IL-2 in human rabies, a significant proportion of rabies patients had elevated sIL-2r and IL-2 in their sera. These suggest that other stimulating factors for NK than IL-2 may be lacking. Corticotropin releasing hormone (CRH) has been demonstrated to be important in modulating NK function. It is still not known whether rabies virus preferentially selects hypothalamus as its early target, thus disturbing synthesis and/or the process of release of CRH.

Table 1. Characteristics of patients with rabies

| Patient no. | Clinical type | Age (years) | Sex | Incubation period (months) | Days from onset to admission | Days from admission to death |
|---|---|---|---|---|---|---|
| 1 | Encephalitis | 6 | Female | 0.3 | 3 | 1 |
| 2 | Encephalitis | 33 | Male | 2 | 4 | 2 |
| 3 | Encephalitis | 28 | Male | 4 | 2 | 2 |
| 4 | Encephalitis | 51 | Female | 2 | 3 | 3 |
| 5 | Paralysis | 18 | Male | 2 | 4 | 1 |
| 6 | Encephalitis | 31 | Female | 1 | 3 | 2 |
| 7 | Paralysis | 58 | Female | 5 | 6 | 1 |
| 8 | Encephalitis | 65 | Male | 3 | 5 | 2 |
| 9 | Encephalitis | 23 | Male | 2 | 1 | 3 |
| 10 | Paralysis | 11 | Male | 8 | 4 | 1 |
| 11 | Encephalitis | 7 | Female | 0.5 | 4 | 1 |
| 12 | Encephalitis | 63 | Male | 2 | 4 | 2 |
| 13 | Encephalitis | 52 | Female | 3 | 2 | 2 |

Table 2. Checkerboard titration for the determination of optimal dilution of mouse anti-human CD 56 primary Ab and biotinylated horse anti-mouse IgG secondary Ab

| Cells | 2 Ab (dilutions) | 1 Ab (dilutions) 1:25 | 1:50 | 1:100 | 1:200 |
|---|---|---|---|---|---|
| | | * ** | | | |
| Normal 1 | 1:100 | 16 (MOD) | 15(SL) | 14(NB) | 13(NB) |
| | 1:200 | 16(MOD) | 13(NB) | 13(NB) | 12(NB) |
| Normal 2 | 1:100 | 15(MOD) | 14(SL) | 14(NB) | 12(NB) |
| | 1:200 | 14(SL) | 14(SL) | 12(NB) | 12(NB) |
| Normal 3 | 1:100 | 16(MOD) | 16(SL) | 15(NB) | 11(NB) |
| | 1:200 | 17(MOD) | 16(SL) | 15(NB) | 12(NB) |

* Percentage of positive cells

** Background : MOD = moderate, SL = slightly, NB = no background

Table 3. Checkerboard titration for the determination of optimal dilution of mouse anti-human CD 57 primary Ab and biotinylated horse anti-mouse IgG secondary Ab

| Cells | 2 Ab (dilutions) | 1 Ab (dilutions) 1:25 * ** | 1:50 | 1:100 | 1:200 |
|---|---|---|---|---|---|
| Normal 1 | 1:100 | 18 (MOD) | 17(NB) | 14(NB) | 13(NB) |
| | 1:200 | 17(SL) | 15(NB) | 14(NB) | 12(NB) |
| Normal 2 | 1:100 | 19(SL) | 18(NB) | 16(NB) | 11(NB) |
| | 1:200 | 19(SL) | 16(NB) | 14(NB) | 11(NB) |
| Normal 3 | 1:100 | 17(MOD) | 15(NB) | 14(NB) | 13(NB) |
| | 1:200 | 17(MOD) | 15(NB) | 12(NB) | 13(NB) |



* Percentage of positive cells

** Background : MOD = moderate, SL = slightly,
NB = no background

Table 4. Number of cells with CD 56 and CD 57 phenotype in PBMC of controls and rabies patients.

| | % CD 56 | % CD 57 |
|---|---|---|
| | * ** | |
| Normal (n=31) | 11-18 (14.5±1.8) | 14-21 (17.0±1.9) |
| Rabies (n=13) | 10-18 (14.3±2.4) | 4-12 (7.9±2.3) |
| | (p > 0.05) | (p < 0.001) |

* Range

** $\bar{X}$±SD

Table 5. Activity of cells with natural killing function in lytic unit(LU) / $10^7$ cells of PBMC of controls and rabies patients.

| | Activity |
|---|---|
| Normal (n=31) | 55.4 ± 22.3 * |
| Rabies (n=13) | 45.3 ± 18.8 ($p > 0.05$) |

* $\bar{X} \pm SD$

ENCEPHALITIC RABIES

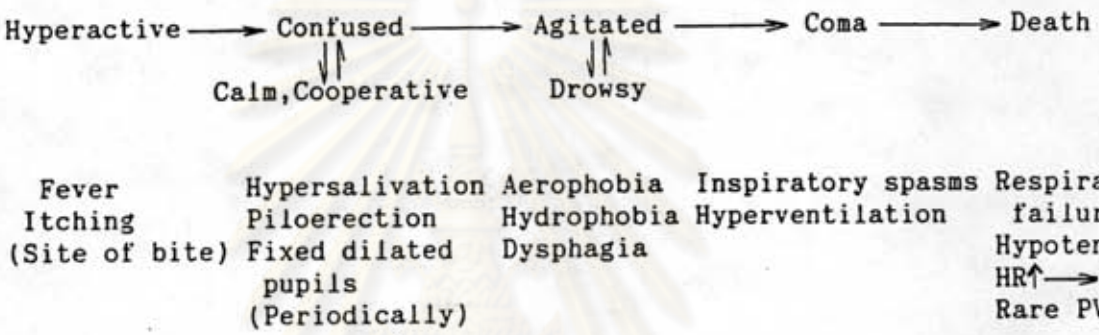


Figure 1. Clinical pattern of human encephalitic rabies

PARALYTIC RABIES

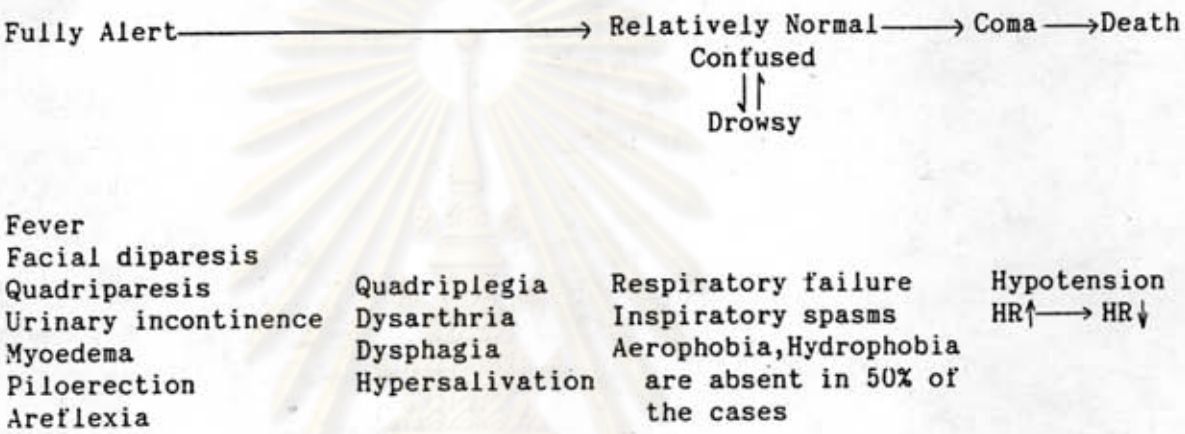


Figure 2. Clinical pattern of human paralytic rabies.

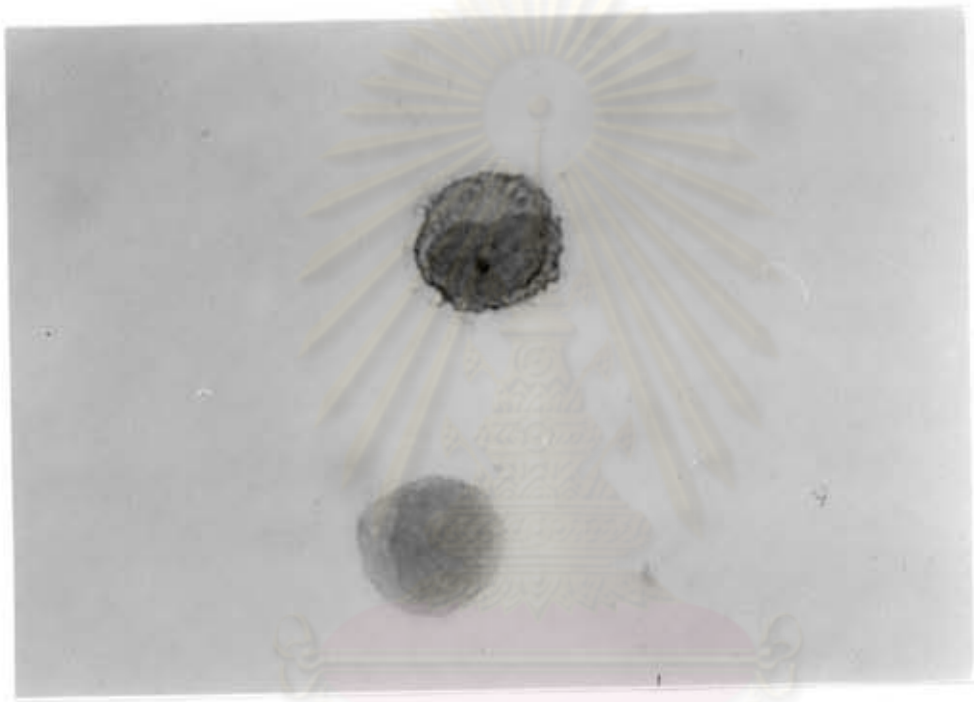

Figure 3. Immunoperoxidase staining of cells with CD 56 or CD 57 phenotype in PBMC showing brown diffuse cytoplasmic staining (x 100).

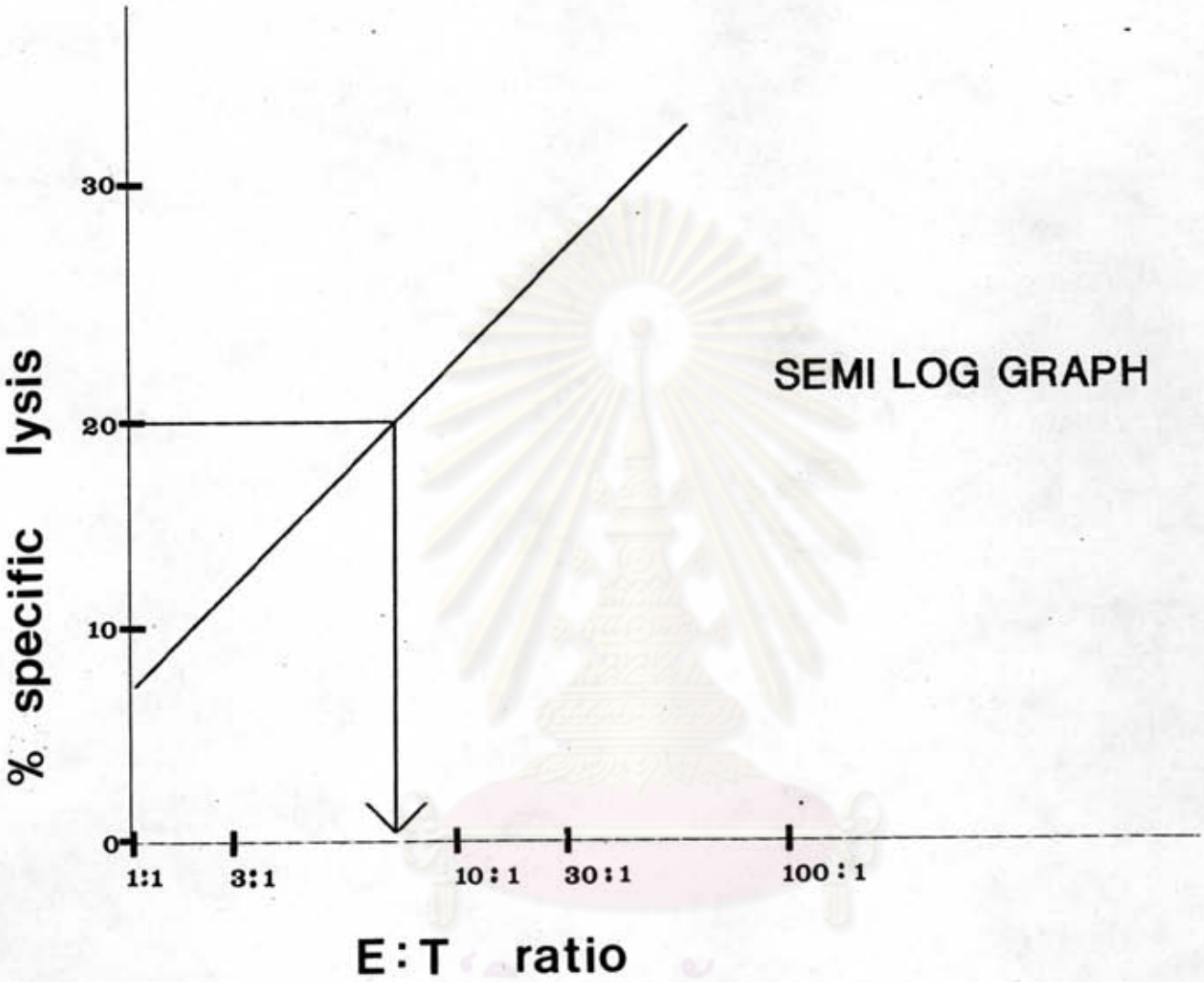


Figure 4. Least squares analysis for the determination of lytic unit (LU) : one LU was defined as the number of effector cells required for 20% specific lysis of $5x10^3$ target cells.

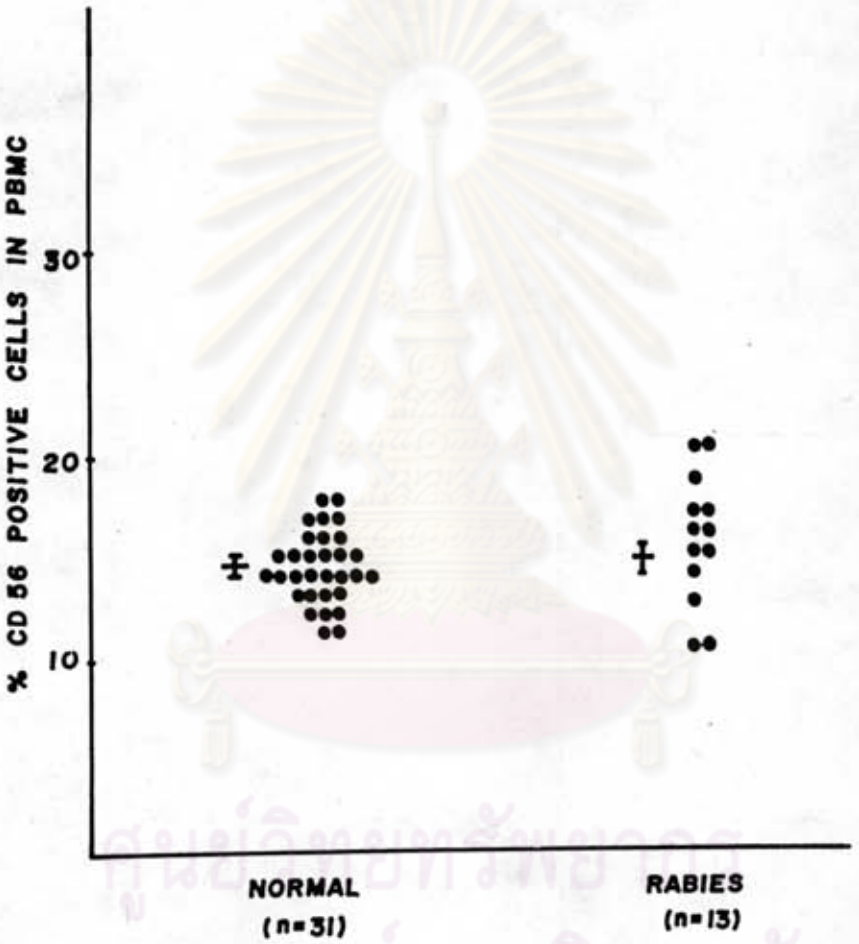


Figure 5. Percentage of CD 56 positive cells in PBMC from individuals with rabies and controls.

$\dagger$ $\bar{X} \pm$ SEM

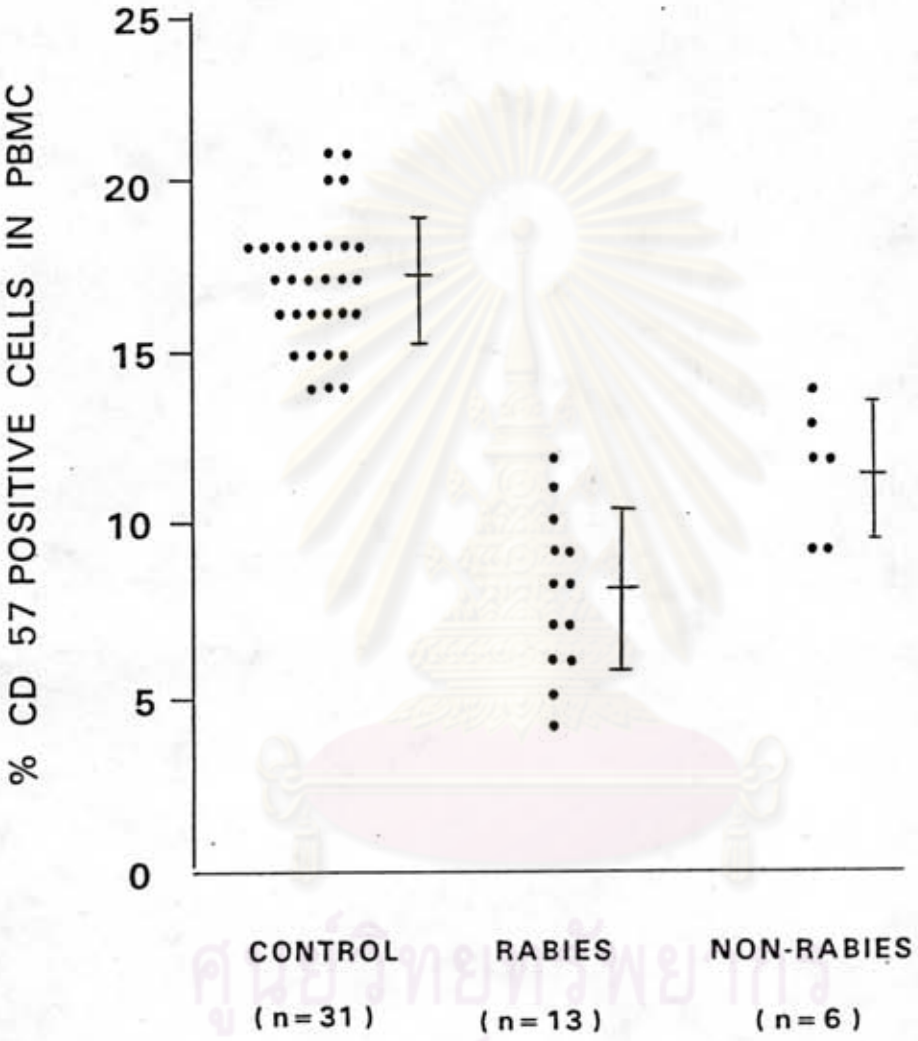


Figure 6. Percentage of CD 57 positive cells in PBMC from individuals with rabies, non-rabies encephalitis, and controls.

$\bar{X} \pm$ SEM

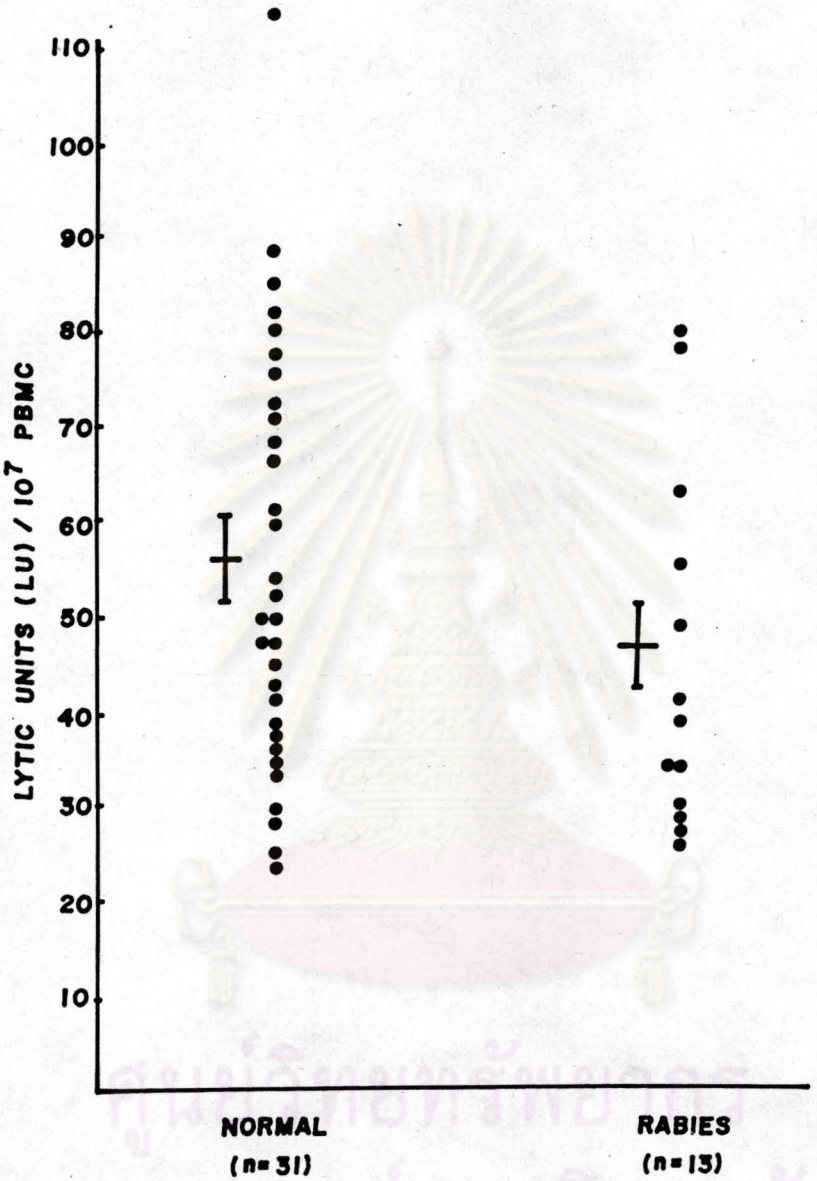


Figure 7. Activity of cells with natural killer function in lytic units / $10^7$ cells of PBMC from individuals with rabies and controls.

$\bar{X} \pm$ SEM

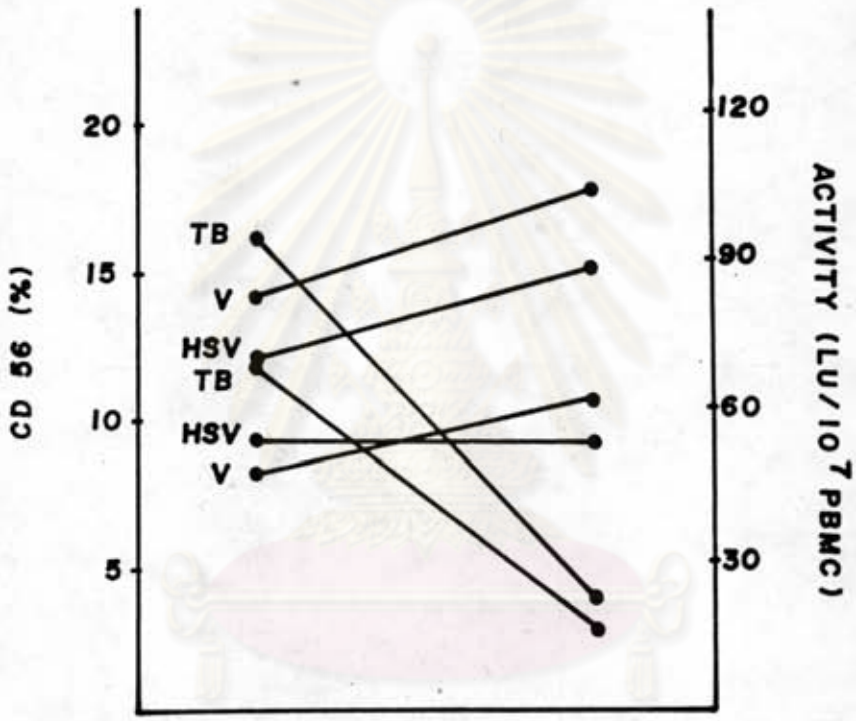


Figure 8. Percentage of CD 56 positive cells and NK activity in non-rabies encephalitic patients.

TB = Tuberculous Meningoencephalitis

V = Unidentified Viral Encephalitis

HSV = Herpes Simplex Encephalitis

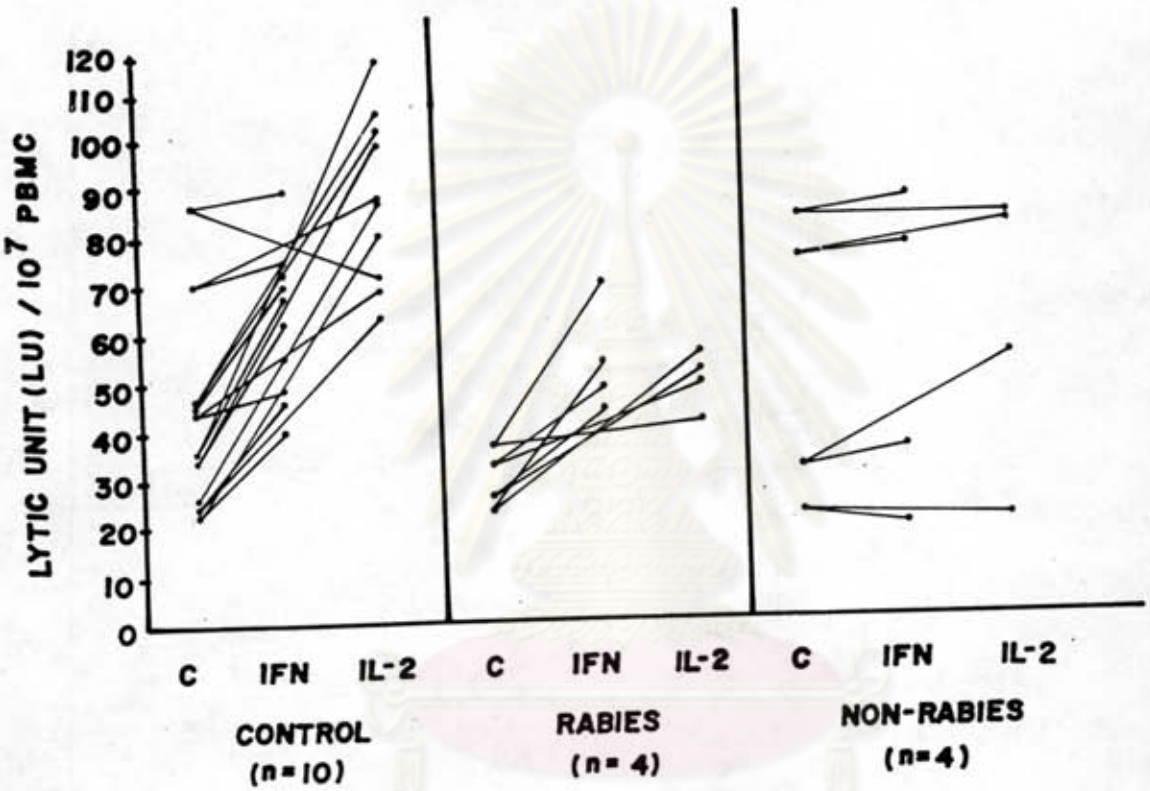


Figure 9. Activity of cells with natural killing function in lytic units / $10^7$ cells of PBMC from individuals with rabies, non - rabies encephalitis, and controls cultured overnight without additives (C), with 100 units recombinant $\alpha$ -interferon (IFN) or 100 units recombinant interleukin-2 (IL-2).