Localization of Intracellular Monoclonal Antibody Specific for Mycobacteria in Experimentally Induced Pulmonary Tuberculosis

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Purpose: To determine whether intracellular localization of intravenously injected infection specific MoAb can be localized in the infected cells, immunohistochemical staining was obtained in animals infected with Mycobacterium tuberculosis.

Materials and Methods: One mg of intact mouse MoAb against mycobacteria (group I, n=10) and F(ab')2 (group II, n=6) were intravenously injected to the rabbits of each groups infected with M. tuberculosis H37RV. Immunohistochemical staining using an anti-mouse antibody was obtained at the 1, 3, 5, 7 and 8th days in Group I and at the 1, 2, 3rd days in group II by streptavidin-biotin method. For the control study, 1 mg of non-specific polyclonal human IgG (group III, n=10) and 100μg of normal rabbit IgG F(ab')2 (group IV, n=6) were injected to the rabbits and guinea pigs respectively.

Results: Both groups (group I & II) showed a positive Ag-Ab reaction within the cytoplasm of monocytes. A weak but positive reaction was observed intracellularly in group II, however no positive reaction in group IV.

Conclusion: Our results suggest intracellular Ag-Ab reaction plays an important role in the localization of infection by immunoscintigraphy using a specific MoAb fragments.

Index Words: Tuberculosis, pulmonary
Lung radionuclide studies
Monoclonal antibodies

INTRODUCTION

It is critical for the management of an infectious process to localize the site and extent of focal inflammation and to identify a causative micro-organism. Immunoscintigraphy has continued to be used and investigated for these purposes. Although immunoscintigraphy using a non-specific human polyclonal IgG (HIG) has been used (1-3), it lacks specificity for a causative micro-organism. Also, the mechanism of localization at the infection site is currently under investigation. Morrel and his colleagues reported the accumulation of In-111-HIG within the expanded interstitial spaces without intracellular deposition by microautoradiography (4), suggesting increased permeability as a major mechanism of IgG localization. In addition, increased influx rate of IgG with reduced efflux rate in inflammatory site is also thought to be an important mechanism (5).

In our earlier report, we attempted an infection specific immunoscintigraphy using I-131-polyclonal antibody against Mycobacterium bovis bacillus Calmette-Güerrin (BCG) which accumulated in experimentally induced tuberculous foci but the antibody rapidly washed out from the non-tuberculous foci (6). It is also observed, however, even non-specific Ab including F(ab')2 is retained in the tuberculous site. Therefore it remained to be solved whether specific targeting of the micro-organism with the specific antibody is possible (7). The purpose of this study is to...
demonstrate specific Ag-Ab reaction within the infected cells in the inflammatory site by immunohistochemical staining.

**MATERIALS and METHODS**

**Induction of Pulmonary Tuberculosis in Animals**

Pulmonary tuberculosis was developed in 26 New Zealand white rabbits weighing about 2.5–3.0 kg and in six guinea pigs weighing around 300 g either by intravenous injection of heat-killed lyophilized Mycobacterium tuberculosis H37Rv (3 × 10^9 bacilli) via ear vein or inoculation of bacilli by direct puncture of the lung under the fluoroscopic guide (6). Direct puncture of the lung was done in several animals especially in the guinea pigs in which venous access was not available. The tuberculous infection was allowed to develop for two weeks.

**Antibody Production and Preparation**

MoAb specific for mycobacteria was produced as described previously (8). In brief, BALB/C mice were immunized with Mycobacterium leprae having lipoarabinomannan-β which has been known for a long time as a mycobacterial common antigen. The M. leprae were mixed with Freund’s incomplete adjuvant (Sigma Chemical Co., St. Louis, Mo). Three weeks after the injection, booster injection was given and after giving another booster four days later, the spleen was removed and the spleen cells were fused with Sp2/O-Ag-14 mouse myeloma cells. The colonies producing anti-Mycobacteria Ab were cloned and IgG MoAb was obtained.

The F(ab')2 of the MoAb was prepared by pepsin digestion in pH 4–4.5 acetate buffer at 37°C overnight and purified by size-exclusion chromatography using a Sephacryl S-200 (Pharmacia, Uppsala, Sweden). HIG was obtained from Green Cross Corp (Seoul, Korea) and control rabbit F(ab')2 was obtained from Dakopatts (Glostrup, Denmark).

**Animal Experiments**

One mg of intact MoAb against mycobacteria diluted in 10ml of normal saline was injected intravenously to group I (n=10) and same dose of MoAb F(ab')2 was given to group II (n=6). For the control study, 1mg of HIG for intravenous use was injected to group III (n=10) and 100μg of normal rabbit F(ab')2 was given to group IV (n=6). Animals used in group I–III were rabbits and in group IV guinea pigs were used. The reason we chose guinea pig as a model in group IV is to avoid the cross reactivity of the given rabbit F(ab')2 with the Abs within the host animals. The dose of rabbit F(ab')2 in guinea pigs was normalized by the weight; 100μg of F (ab')2 was injected as the weight of guinea pig was one-tenth of the rabbits.

At 1, 3, 5, 7 and 8 days after the injection of intact MoAb or HIG and at 1, 2 and 3 days after the injection of MoAb F(ab')2 or control F(ab')2, all animals were subjected to the pathologic examinations including Hematoxylin–Eosin (HE), Ziel-Neelson and immunohistochemical stains.

**Immunohistochemistry**

The specimens were fixed in 10% formalin for 24 hours and paraffin embedding was followed. Specimens were cut into 5μ thickness and mounted on glass slides. After deparaffinization in an oven at 58°C, each sections were gradually hydrated with alcohol for five minutes. After blocking of endogenous hydrogen peroxide, blocking serum (swine serum diluted to 20% in 0.05M Tris-HCl buffer in pH 7.2–7.6) was applied to the specimens and incubated for 10 minutes. Rabbit antimouse IgG for Group I and II, mouse anti-human IgG for Group III and mouse anti-rabbit IgG for Group IV were used as a primary antibody for immunohistochemistry.

Biotinylated secondary antibodies (anti-rabbit Ab for group I and II, anti-mouse Ab for group III and IV) were applied and incubated with streptavidin reagent. All of the immunochemicals were obtained from Dakopatts (Glostrup, Denmark). Three- amino-9-ethylcarbazole (AEC), (Biomeda corp, Foster city, USA) was used for chromogen and the specimens were counter stained with hematoxylin.

For the negative control of immunohistochemistry, the specimens were stained with irrelevant MoAb (mouse anti-CD3 Ab), with the same method.

To confirm the presence of tuberculous antigens in inflammatory cells within the tuberculous foci, immunohistochemistry was also performed with a rabbit polyclonal antibody against M. bovis BCG (Dakopatts, Glostrup, Denmark) by avidin-biotin method.

**RESULTS**

All animals demonstrated tuberculous inflammatory reactions in the lungs. The histologic examination showed the granulomatous lesions infiltrated by many monocytes. Immunohistochemical staining using an anti-BCG Ab revealed positive Ag-Ab reactions within the cytoplasm of histiocytes. The positive reaction within the cells containing AFB was stronger than that within the cells without AFB (Fig. 1). The granulomatous lesions induced by direct puncture were larger in size than those induced by intravenous injection of bacilli. However, two methods produced on identical pathologic features.

The group I, injected with the intact MoAb specific for mycobacteria, demonstrated strong positive reactions almost exclusively within the cytoplasm of monocytes upto 8th days post injection. The pattern of positive reaction was no different from that of the specimen.
obtained at the first day of injection. The distribution of cells with positive reaction was inhomogeneous, mainly at the periphery of the granulomatous lesions sparing the central area (Fig. 2).

The group II, injected with the MoAb F(ab')2, also showed positive reaction within the cytoplasm of monocytes. The distribution pattern of cells with positive reaction was same as in the group I (Fig. 3).

The group III, injected with non-specific intact HIG, demonstrated positive reaction within the interstitial space as well as within the inflammatory cells although intensity was much less. The number of positive cells was fewer than those in group I or II (Fig. 4).

The group IV, injected with non-specific rabbit F(ab')2 did not show any positive reaction within the cytoplasm even in the first post injection day (Fig. 5).

Fig. 1. Immunohistochemistry using a rabbit polyclonal antibody against M. bovis BCG counterstained with Ziel-Neelson stain in a tuberculous lesion. Positive reaction is seen within the cytoplasm of histiocytes (brown color, ×400), especially in the cells containing AFB (arrows in inset, ×1000). The distribution of Ag positive cells is rather homogeneous.

Negative control immunohistochemistry using an irrelevant antibody did not reveal any positive reaction.

Fig. 2. Immunohistochemistry 5 days after the injection of intact MoAb against mycobacteria shows positive reaction within the cytoplasm of monocytes mainly along the periphery of granuloma (×400).

Fig. 3. Immunohistochemistry, 48 hours after the injection of MoAb F(ab')2 shows strong positive reaction within the cytoplasm of monocytes (×400).

Fig. 4. Immunohistochemistry 5 days after the injection of HIG shows weak positive reaction within the cytoplasm of monocytes and some PMNs (arrows) (×400).

Fig. 5. Immunohistochemistry 48 hours after the injection of normal rabbit F(ab')2 in a guinea pig shows negative reaction (×400).
DISCUSSION

Immunoscintigraphy using a tumor-associated antibody is being successfully utilized in the diagnosis and the treatment of solid tumors. However, a few investigations were reported on immunoscintigraphy using infection-specific antibodies. Recently, Malpani (9) and Lee et al. (6) performed animal experimental studies to detect tuberculous lesion using a radiolabeled tuberculosis specific antibody. These reports showed promising results and demonstrated a feasibility of diagnosing a specific infectious process, although the principal physiologic mechanisms of the antibody localization remained to be solved (7). Since M. tuberculosis is an intracytoplasmic organism, the intracellular deposition of the given tuberculosis specific MoAb should be demonstrated within the cytoplasm in order to confirm the presence of Ag-Ab reaction.

Our study showed that both intact and fragmented anti-mycobacteria MoAb were deposited intracellularly, which indicates the intracellular localization of tuberculosis specific antibody is mediated by a Ag-Ab reaction.

The internalization of the antibody within the cells may be the results of the receptor-mediated endocytosis, a well-known mechanism especially in the MoAbs targeting the lymphoid and myeloid antigens (10-12). In tuberculosis, we assumed the complement activation and resultant increased phagocytosis, increased blood flow to the site of activation and capillary permeability facilitated the internalization process of antibody. Phagocytosis is most active when both elements of the activated complements and specific antibodies are present by binding of the Ag-Ab complex and complement systems to the receptors of phagocytic cells (13-14). In addition, the penetration of the specific Ab through abnormally permeable cell membranes has also been reported as a possible mechanism for the internalization of Ab (15-16). However, non-specific F(ab')2 cannot be internalized since it is not able to activate the complement system nor it can induce Ag-Ab complex (14).

In contrast to the specific antibody, Morrel et al. demonstrated the In-111-labeled non-specific HIG localized within the expanded interstitial space by microautoradiography (4). Their finding is somewhat contradictory to our results since positive reaction, although which is less intense than that of MoAb, was observed within the cytoplasm of inflammatory cells. The internalization of HIG is presumably due to IgG aggregation. The IgG polymer could be made by myeloperoxidase and hydrogen peroxide released by oxygen dependant cell killing mechanism of leukocytes (17-18). The IgG polymers can be internalized by phagocytes.

In terms of the methods demonstrating the distribution of radiolabeled antibody in tissue, a microautoradiography is a preferred method. However, In-111 may be dissociated from the antibody and hydrolysed to be highly insoluble (19). Therefore, autoradiography might not reflect the exact location of Ab.

Although the immunohistochemistry is effective in the demonstration of intracellular localization of antibody, it also has limitations. Immunohistochemistry using a paraffin embedded section may not detect the surface bound or extracellular distributed IgG (19). Thus the fact that weak positive reaction of the HIG within the cytoplasm of inflammatory cells observed in group III may indicate that majority of HIG was distributed within the extracellular interstitial space. However, a certain proportion of HIG was associated with inflammatory cells. Another limitation of our study is difficulty in the quantification of Ab concentration since specimens were obtained from different animals with various degrees of inflammatory response and the antibody concentration depends on the severity of inflammation (7). Nevertheless, the main objective of this study was to demonstrate the intracellular localization of specific antibody.

We concluded that infection specific antibody did localize within the inflammatory sites by the Ag-Ab reaction in a greater degree than that of non-specific antibody. On the basis of our experimental study, immunoscintigraphy using a radiolabeled specific antibody fragments against M. tuberculosis might be effective in the localization of tuberculosis more specifically and in the evaluation of tuberculosis activity.

REFERENCES

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 실험동물에서 유발시킨 폐결핵 병변의 세포내 결핵균에 대한 단세포균 항체의 국소화

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목적: 실험동물에서 유발시킨 폐결핵 병변의 세포내 결핵균에 대한 단세포균 항체국소화를 도모하였다.

대상 및 방법: 약 2.5~3.0kg의 무게를 갖는 가토 26마리와 300g의 guinea pig 6마리에 결핵균 (M. tuberculosis H37Rv)을 정맥주사 혹은 폐조직에 주입하여 폐결핵 병변을 유발시켰다. 각 동물은 4군으로 나누어 저1 1군( n = 10 )은 결핵균에 대한 항체, 제 2군(n = 10 )은 항체의 분절 [F(ab')2], 제 3군 (n = 10 )은 결핵균과 반응하지 않는 인면역글로불린, 제 4군 (n = 6 )은 정상 가토의 면역글로불린 분절을 정맥주입하여 시간별로 주입한 항체에 대한 이차 항체를 이용하여 면역조직학염색을 시행하였다.

결과: 저1 1군과 2군에서는 주입된 항체가 결핵균에 국소화된 세포내에 침착되는지의 여부를 보고 위하여 결핵균에 감염된 실험동물에서 면역 및 조직학적 혈을 이용하여 이를 증명하고자 하였다.

결론: 결핵균에 대한 단세포균 항체를 주입하면 항원-항체 반응에 의하여 결핵균의 세포내에 침착된 것을 알 수 있었다.

본 연구는 연세대학교 의과대학의 승인을 받아 수행된 결과로, 실시에 참가한 동물의 실수상 해로 인한 교유적인 피해를 사과한다.
**Case 1. M/47**

C.C.: epistaxis since 10 days ago

Answer: sinonasal hemangioma