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Protein composition and antigenicity of the tegument from *Paragonimus westermani*

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Abstract: To ascertain that tegument of *Paragonimus westermani* has specific antigenic proteins, the tegumental fraction was isolated from 10-month-old worms by 0.1% digitonin solution, and subjected to SDS-PAGE and immunoblot. Component proteins of tegumental syncytium comprised of 94, 74 (76-66), 62, 54, 44, 42, 38, 28, 26, 25, 24, 17, 15.5 and 13.5 kDa proteins. Of them, the 94, 44 and 42kDa proteins were more specific to tegument, especially the 94 kDa protein was the most prevailing one. In immunoblot, antigens of the 94, 90, 78, 76, 74, 68, 65, 62, 60, 59 and 54 kDa proteins were commonly detected by 7 sera of 10 human paragonimiasis, but none of them reacted with 5 sera of clonorchiasis. In conclusion, the 94 kDa protein was the major tegumental protein, as well as the specific antigen. The 76 and 66 kDa proteins were the minor components of tegument, which were also specific antigens of *P. westermani*.

Key words: *Paragonimus westermani*, tegument, antigenicity, immunoblot

INTRODUCTION

Antigenic localization of *P. westermani* has been unveiled by adoption of advanced immunological methods. Immunohistochemical staining of adult *P. westermani* with polyclonal antibodies ascertained that antigenic sites were intestinal epithelial border, luminal contents, eggs both in uterus and around worm capsule, vitelline glands, and parenchymatous portion of the worm (Sugiyama, 1987; Lee *et al.*, 1989; Kong *et al.*, 1992). By using homologous monoclonal antibodies in immunostaining or indirect fluorescent antibody technique, the intestinal border, the eggs in lung granuloma, the parenchymal tissue, and the vitelline follicles of *P. westermani* were also confirmed as antigenic foci (Sugiyama *et al.*, 1988; Kang *et al.*, 1991; Yong *et al.*, 1993). Furthermore, immunogold labeling method make it possible to scrutinize the antigenic localities in ultrastructural level. In adult *Paragonimus*, secretory granules in the parenchymal tissue, cytoplasm between granules in the vitelline glands, the epithelial lamellae of caeca were positively immunogold labeled. In the juvenile worm of 4 weeks old, on the while, the gold particles appeared in the tegumental syncytium, but disappeared after 8 weeks (Kwon *et al.*, 1991; Rim *et al.*, 1992).

Based on these findings, it was assured that the representative antigens of *P. westermani* were present in the eggs, the intestinal epithelial border and intraluminal contents, the vitelline glands and the parenchymal tissue, whereas the tegument had no or very weak antigenicity. Contrary to this, however, it has been generally known that the tegument of

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trematodes was a metabolically active layer, and one of its roles was the synthesis and secretion of antigenic materials. And the secretory product of tegument might be involved in the immunological relationships between parasite and host (Smyth and Halton, 1983). In this respect, lots of studies have been done in *Schistosoma mansoni* on the tegumental or surface antigens (Simpson *et al.*, 1981; Taylor and Wells, 1984; Payares *et al.*, 1985).

In this context, it seems necessary to reevaluate the tegumental antigenicity in *P. westermani*. This study was undertaken to observe antigenicity in isolated tegument preparation from the worm.

MATERIALS AND METHODS

1. The parasite and sera: Ten-month old adult worms of *P. westermani* were recovered from a dog which was experimentally infected with metacercariae from naturally-infected crayfishes. The recovered worms were washed with normal saline and kept overnight at 4°C.

The sera of paragonimiasis consisted of 6 egg-positive and 4 serologically-positive human patients of *P. westermani* infection. The sera of clonorchiasis were from 5 egg-positive cases of *Clonorchis sinensis* infection.

2. Isolation of the tegumental fraction: According to the method of Mills *et al.* (1984), 80 worms were incubated for 20 minutes with gentle agitation in 40 ml of 0.1% (w/v) solution of digitonin (Sigma Co., U.S.A.). The digitonin solution with the worms became turbid and gray yellowish. After the incubation, the fluid was recovered, and the worms were again incubated in 10 ml of 0.85% saline for 10 minutes. The supernatant fluid was recovered and combined with the previous one. The combined fluid was centrifuged 2,500 *g* for 10 minutes at 4°C. The 2,500 *g* pellet, the tegumental fraction, was washed twice with normal saline, suspended in 1 ml distilled water, and freeze-dried.

3. Extraction of tegumental proteins: The lyophilized tegumental fraction was treated by sonication with 1 ml of lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris, 1.0 mM PMSF), and centifuged 12,000 *g* for 15 minutes at 4°C. The 12,000 *g* supernatant was designated as the tegumental extract of *P. westermani* (PwT). The crude worm extract of *P. westermani* (PwW) was 12,000 *g* supernatant of the homogenate of 10-week-old worms which were homogenized in normal saline using a power-driven Potter-Elvehjem glass-Teflon homogenizer at 4°C.

4. Protein analysis by gel electrophoresis: PwT and PwW were subjected to sodium dodecyl sulfate polyacrylamide gel electophoresis (SDS-PAGE) according to the method of Laemmli (1970). The electrophoresis apparatus was Protean II Slab Cell (Bio-Rad, USA). The 4.0% stacking gel and 10-15% linear gradient separating gel were prepared with 1.5 mm gel thickness and run under the constant current of 35 mA. The component proteins in the gel were detected by silver staining kit (Bio-Rad, USA), and their molecular weights (kDa) were estimated according to the calibration line of marker proteins (Sigma Co., U.S.A.).

5. Antigenic characterization by immunoblot: After the SDS-PAGE of PwT, the immunoblot was performed according to the method of Tsang *et al.* (1983) with some modifications. The electrophoretic transfer was done using Trans-Blot Cell (Bio-Rad, USA). The proteins were transferred from the gel to nitrocellulose (NC) sheet (Sigma Co, U.S.A.) in the transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) under the constant 30 V for 18 hours. After the transfer, NC sheet was blocked with 3% bovine serum albumin in Tris buffered saline (BSA/TBS) for 1 hour. NC strips were reacted for 18 hours with the sera diluted to 1:100 in 0.5% BSA/TBS. After the washing with TBS, they were incubated for 3 hours with the 1:1,000 diluted conjugate of the peroxidase-conjugated anti-human IgG (H+L chains specific) (Cappel, U.S.A.). The final substrate reaction was completed with 0.05% 3,3'-diaminobenzidine (Bio-Rad, U.S.A.) and 0.03% (v/v) H_2O_2 solution.

6. Electron microscopy: Ten-month-old *P. westermani*, before and after tegumental

isolation, were washed with normal saline, fixed in 2.5% glutaraldehyde, and refixed in 1.0% osmium tetroxide. After dehydration in ethanol series, for the transmission electron microscopy (TEM), they were embedded in Epon mixture, sectioned, and examined with JEM-100CXII (Jeol Co.). For the scanning electron microscopy (SEM), the dehydrated samples were freeze-dried, gold-coated, and examined with ISI SS-60 (ISI Co.).

RESULTS

1. Electron microscopic observation: The electron microscopic findings of *P. westermani* which had been incubated in the nonionic detergent solution of digitonin revealed that the distal tegument was missing. The surface of carcass after the digitonin treatment was wrinkled with many grooves (Fig. 1). As shown in Fig. 3, the tegumental syncytium was not observed, but the basal lamina, the muscles, and the parenchymal tissue were present, although they looked less dense than those of normal worm. Compared to this, a 10-month-old normal *P. westermani* showed the distal tegument which had spinal projections and rugae on the surface (Fig. 2), and revealed the characteristic morphological features (Fig. 4).

2. Protein composition of the tegumental fraction: When the PwT was solubilized in the SDS and separated by PAGE, the 94, 74 (76-66), 62, 54, 44, 42, 38, 28, 26, 25, 24, 17, 15.5 and 13.5 kDa protein bands were detected by silver staining (Fig. 5). Protein bands of 94, 44, 42, 38, 28, 26 and 24 kDa were observed only in PwT. Of them, the 94 kDa band was the most prominent protein. In addition, the 44 and 42 kDa bands were stained more prominently than others.

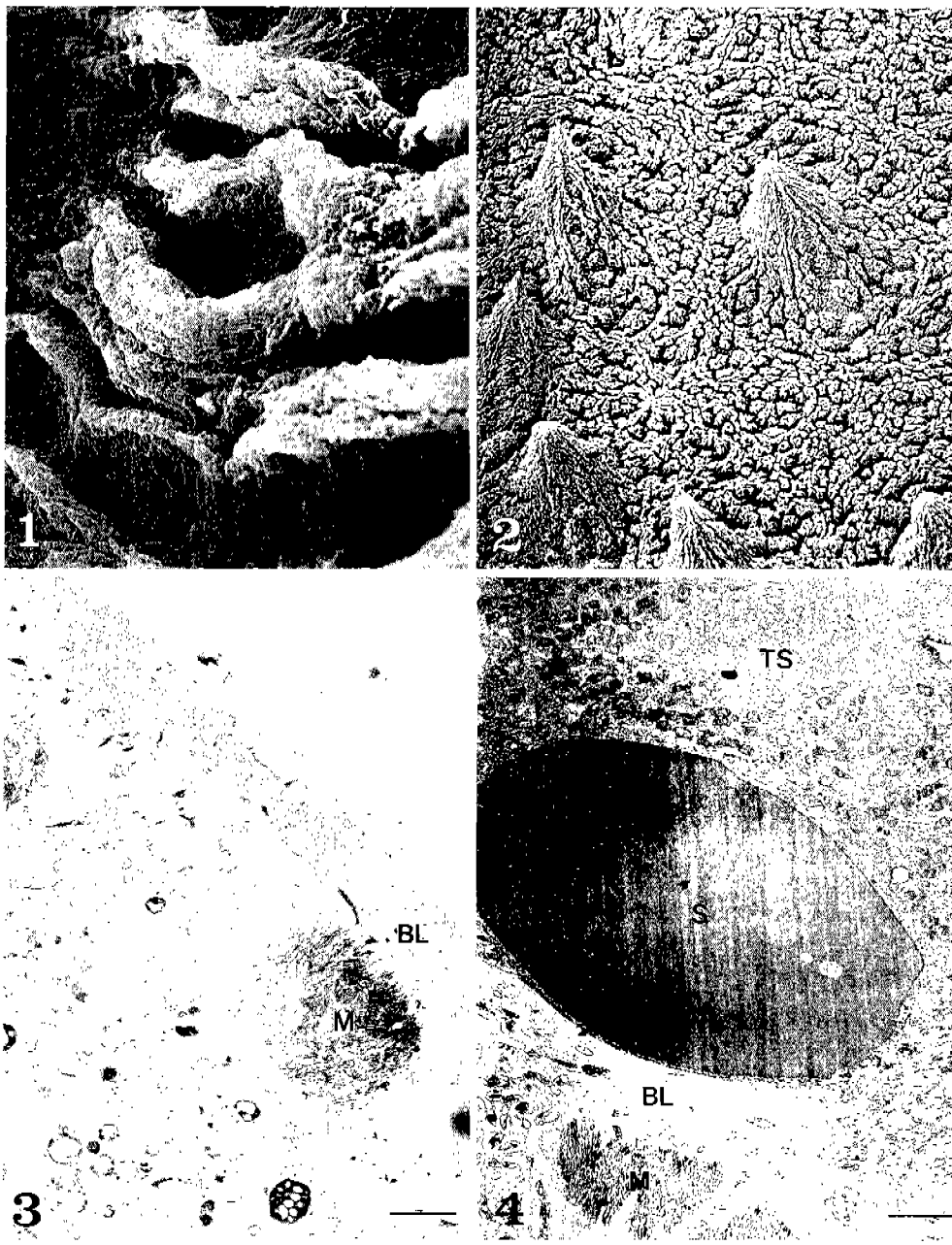
3. Antigenicity of the tegumental proteins: As shown in Fig. 6, the immunoblot of the tegumental proteins, probed by human sera of paragonimiasis, revealed 11 antigenic bands of 94, 90 (group A), 78, 76, 74 (group B), 68, 65 (group C), 62, 60, 59 (group D) and 54 (group E) kDa which were grouped by their size. The antigens of groups A to E were compatible with the 94, 76, 66, 62, 54 kDa bands of PwT, respectively. All the 5 groups of antigen bands reacted with sera of 7 cases out of 10 paragonimiasis patients; but no reactions were observed in 5 sera of clonorchiasis to the above antigen groups. The group A, B and C matched with the 94, 76 and 66 kDa proteins of PwT were more strongly reacted with sera of paragonimiasis than the group D and E. The protein bands of PwT below 44 kDa were not detected in immunoblot when reacted with the sera of paragonimiasis or clonorchiasis.

DISCUSSION

Many detergents are available for the isolation of the surface tegument of helminth parasites; digitonin (Mills *et al.*, 1984), saponin (Kusel, 1972) and Triton-X (Oaks *et al.*, 1981) have been used to disrupt and remove the tegument. In this study, the 0.1% digitonin-saline solution was used to isolate the tegumenal syncytium of *P. westermani*, because this nonionic detergent was verified to yield the most intact tegumental fraction with the least amount of contamination (Mills *et al.*, 1984).

By digitonin treatment of *P. westermani*, the clear digitonin solution became turbid and gray yellowish. The change to turbid solution was due to the disrupted tegument fragments, as judged from electron microscopic findings. The tegumental syncytium was evidently detatched from the worm. In addition to the tegument, the damaged muscles and the parenchymal tissue seemed to contribute to the yellowish color of the solution, because the tissue fluid or parenchymal proteins can possibly ooze through damaged tissues of the worm. In fact, the 2,500 *g* supernatant of digitonin-extracted solution contained a large amount of soluble protein, and it deserves further studies on antigenic nature.

Based on the present experiment, the lyophilized tegument of *P. westermani* was hardly homogenized by Potter-Elvehjem glass-Teflon homogenizer, but easily solubilized in the detergent-contained lysis buffer by sonication. This finding suggests that some proteins in PwT were membrane-bound types including the major tegumental protein of 94 kDa. This protein was observed prominently in



Figs. 1-4. Electron microscopic morphology of *P. westermani* after and before 0.1% digitonin incubation. **1.** Scanning electron microscopy (SEM) of 10-month-old worm after removal of tegument showed many grooves on the surface (× 1,480, 30 kV). **2.** SEM of the normal control worm (× 1,160, 10 kV). **3.** Transmission electron microscopy (TEM) of carcass showed no tegumental syncytium (TS) with spine (S) and less dense structures of basal lamina (BL) and muscles (M) (× 4,000, a bar equals 2.5 μm). **4.** TEM of normal control worm (× 3,500, a bar equals 3 μm).

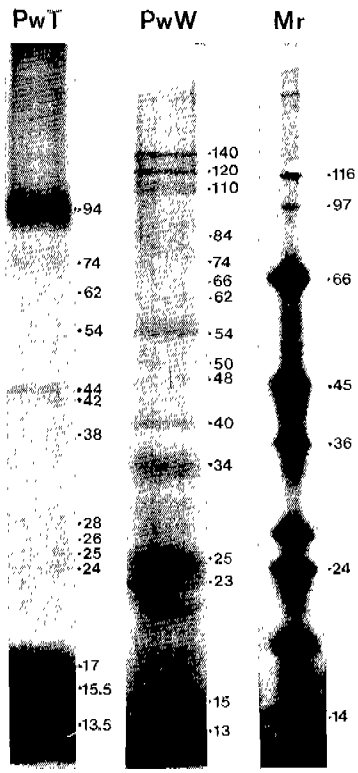


Fig. 5. Protein composition of tegumental fraction of *P. westermani* as observed by SDS-PAGE. Electrophoretic profile of tegumental protein extract (PwT) and that of crude worm extract (PwW) were compared. Each sample was loaded in protein amount of 40 μg to 10-15% linear gradient gel. Protein bands were detected by silver stain. M_r was standard marker proteins, and numerals represented molecular masses in kDa.

electrophoregram of PwT but hardly observed in PwW and other reports on SDS-PAGE findings of the crude whole worm extract of *P. westermani* (Kim *et al.*, 1988; Joo *et al*, 1989a&b; Kim, 1992a). We think that the difference, presence or absence of 94 kDa band, is originated from the different techniques in preparation of the extracts. So far, the 94 kDa protein was recognized when the extract was prepared with detergent and sonication.

The SDS-PAGE of metabolite antigen of *P. westermani*, prepared by simple culture of living worms in physiologic saline, revealed the 62 and 54 kDa proteins. These protein bands may be secretions or tegumental proteins of *P. westermani* (Kim, 1992b). These protein bands are also observed in the profile of PwT. Therefore, the above two proteins could be the secretory tegumental proteins of *P. westermani*.

It is well known that not all the component proteins of *P. westermani* are acting as antigens, and that the major protein bands are not necessarily the strong antigens (Kim *et al.*, 1988; Cho *et al.*, 1989). By the same token, of the tegumental proteins larger than 54 kDa, major bands in SDS-PAGE were not necessarily strong antigens as observed by immunoblot.

A total of 5 tegumental protein bands is observed in the ranges from 94 to 54 kDa by SDS-PAGE. When observed by immunoblot, the antigen-antibody reactions were divided into 11 bands in the range. If each tegumental protein is really a single band in SDS-PAGE, this finding implies that one protein of PwT should have more than one antigenic determinant. On the contrary, it was possible that all the tegumental antigens in immunoblot were not separated into their respective proteins in SDS-PAGE.

The 94 kDa protein is regarded as the most representative tegumental protein of *P. westermani* in this study. In many previous studies on the antigenic proteins of *P. westermani*, species-specific antigen of 91 kDa by Joo *et al.*(1989b), and 92 kDa antigen of Cho *et al.*(1989) may be originated from tegument, for their molecular masses were similar. However, the 92 kDa antigen of *P. westermani* has been stated as egg protein (Kim *et al.*, 1986; Kang *et al.*, 1991; Kong *et al.*, 1992). On the other hand, the 91 or 94 kDa protein appeared in younger worm before egg production (Joo *et al*, 1989a; Kim, 1992a). In this connection, the proteins around 94 kDa, if they are actually the same protein, could have multiple sources of tegument, egg, and other tissues of *P. westermani*. Because the SDS-solubilized polypeptides which had different sources could be observed to have the same molecular mass, two-dimensional electrophoresis will be needed to differentiate the above proteins including the 94 kDa protein of the tegument.

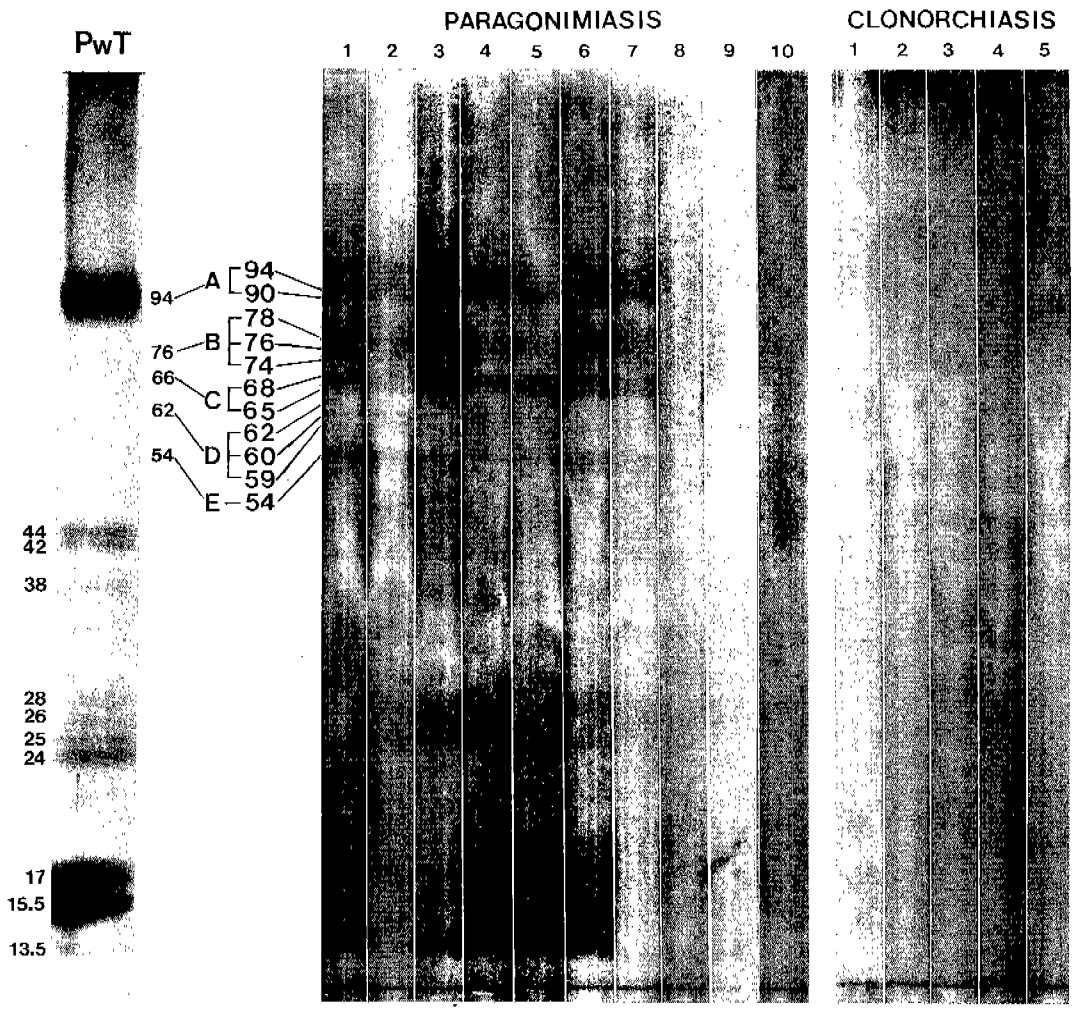


Fig. 6. Antigenicities of tegumental proteins of *P. westermani* as analyzed by immunoblot. Tegumental proteins of 5 groups (from A to E in SDS-PAGE/immunoblot) are antigenic proteins which are matched with proteins in tegumental protein extract (PwT). Their respective antigens reacted with 7 sera out of 10 human paragonimiasis but react with none of 5 sera of human clonorchiasis.

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=국문초록=

폐흡충 충체표피의 단백질 조성 및 항원성

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폐흡충 성충 표피의 구성 단백질의 분자량을 측정하고, 항원성을 갖는 표피 단백질을 규명하였다. 10개월 성충을 0.1% digitonin용액에 담궈 표피하 기저막까지 박리하여 회수한 표피층을 초음파 분쇄하여 표피단백질을 추출하였다. 표피단백질 추출액을 SDS-PAGE와 immunoblot으로 분석하였다. 폐흡충 표피의 구성 단백질은 분자량이 각각 94, 74 (76-66), 62, 54, 44, 42, 38, 28, 26, 25, 24, 17, 15.5, 13.5 kDa으로 측정되었고, 94 kDa 단백질이 가장 주된 표피단백질이었다. 폐흡충 감염자 혈청을 사용한 immunoblot에서 항원성이 확인된 표피단백질의 분자량은 각각 94, 90, 78, 76, 74, 68, 65, 62, 60, 59, 54 kDa 이었다. 이 표피항원 단백질은 폐흡충증 혈청 10개중 7개에서 immunoblot 양성반응을 나타내었고, 7개 양성반응 혈청 각각에서 표피항원 단백질의 특이 항원성이 모두 관찰되었다. 그러나, 이들 폐흡충 표피항원은 간흡충란 양성자 혈청과는 전혀 반응하지 않았다. 이상의 결과로, 폐흡충 표피 단백질중에서 분자량 94-54 kDa 사이의 것들이 폐흡충 특이 항원임을 알 수 있었고, 특히 94 kDa 단백질은 가장 양이 많은 대표적인 표피단백질이면서 아울러 특이항원성도 갖고 있었고, 양이 적은 표피단백질이었던 76, 66 kDa 단백질도 상대적으로 높은 특이 항원성을 보여주고 있음을 확인할 수 있었다.

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