

## ADENOVIRUS TUMOR-SPECIFIC TRANSPLANTATION ANTIGEN IS A FUNCTION OF THE E1A EARLY REGION

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Individual human adenoviruses (Ad)<sup>1</sup> vary in their tumorigenic potential in rodents. Group A serotypes induce tumors with high efficiency, while those belonging to the group C are nononcogenic (1).

This phenomenon was originally thought to be a reflection of differences in their immunogenicity (2). Isograft transplantation experiments have shown, however, that immunization with both highly oncogenic Ad12 (3, 4) and nononcogenic Ad2 and Ad5 (5) serotypes induces a strong transplantation immunity against tumorigenic Ad-transformed cells. This immunity has been attributed to Ad tumor-specific transplantation antigen (TSTA).

The identity of Ad TSTA is unknown. The *in vitro* reactivity of secondary cytolytic T cells against syngeneic Ad-transformed cells is directed against product(s) dependent on expression in the transformed cells of the Ad early region E1 (6, 7). Examination of a series of mutants of Ad5 for their ability to induce group C Ad TSTA in rats revealed that mutants with defects in the region E1 produced significantly lower or no TSTA when compared with Ad2 or Ad5 wild type viruses (5).

Adenovirus early gene block E1 contains the region of the viral chromosome that has been shown to be responsible for the transforming activity of the virus (8). It consists of two transcription units E1A and E1B (9). Effects of immunization with xenogeneic cells transformed with isolated fragments of Ad12 DNA during latency of tumors induced with Ad12 virions suggested that the Ad12 TSTA is a function of the left end of the E1B early region (10). This hypothesis was further supported by evidence that cells transformed with a cloned *Acc* I-H fragment of Ad12 DNA (0–4.7 map units) failed to elicit secondary cytolytic T cells, which were elicited by cells transformed with the *Hind* III-G fragment (0–6.8 map units) of Ad12 DNA (11).

The TSTA immunity is highly specific: immunization with Ad2 (or Ad5) offers

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<sup>1</sup> *Abbreviations used in this paper:* Ad, adenovirus; LIS rats, Lister-Hooded rats; TPD<sub>50</sub>, tumor-producing dose 50; TSTA, tumor-specific transplantation antigen.

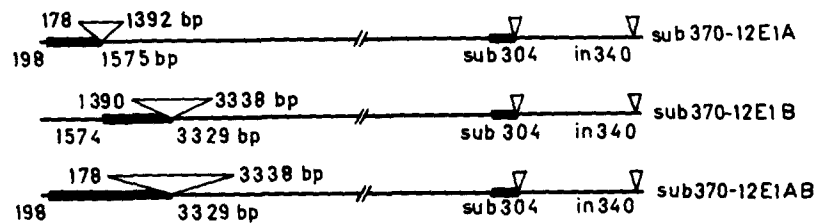


FIGURE 1. Diagrammatic representation of hybrid adenoviruses used for immunization. —, the Ad5 chromosome; ■, deletions; and Δ, insertions. The Ad5 nucleotides present on either side of the Ad5-specific E1 deletions are designated below each chromosome, while the Ad12 nucleotides at the extremes of the Ad12-specific E1 insertions are designated above each chromosome. The *sub304* (14) and *in340* (15) have been described previously.

no protection against Ad12-transformed cells (4) and vice-versa (5). This fact offers an ideal opportunity to identify the adenovirus genes responsible for TSTA expression by use of viable recombinant adenoviruses that carry a portion of the type 12 E1A and E1B transcription units in a type 5 background.<sup>2</sup>

Results we report in this paper do not support the hypothesis that the major component of adenovirus TSTA is coded by the E1B region. We provide experimental evidence that the *in vivo* TSTA activity of the highly oncogenic Ad12 and the nononcogenic Ad5 is a function of their respective E1A genes.

### Materials and Methods

**Animals.** Lister-Hooded (LIS) rats of RT-1<sup>c</sup> haplotype, originally obtained from Dr. Phillip H. Gallimore of the University of Birmingham, England, were bred by brother-sister mating.

**Viruses.** The viable recombinant viruses *sub370-12E1A*, *sub370-12E1B*, and *sub370-12E1AB* were described elsewhere.<sup>2</sup> The genomic structures of these three substitution mutants are diagrammed in Fig. 1. The recombinant viruses, as well as wild type Ad5 and Ad12 (Huie strain), were grown in 293 cells (12).

**Tumor Cell Lines.** Two tumorigenic LIS cell lines were used throughout this study. The EcoC3 cells were transformed *in vitro* with a recombinant plasmid containing the Eco RI-C fragment (0–16.5 map units) of Ad12 DNA. The Ad2/T13 cell line (5) derived from the Ad2/HLREF/50A cell line (13) was kindly provided by Dr. P. H. Gallimore. Both the EcoC3 and the Ad2/T13 cells are tumorigenic in mature syngeneic LIS rats. For tumorigenicity assays the Ad2/T13 cell line was passaged in animals and then once in tissue culture before storage in liquid nitrogen.

**Assays of Transplantation Immunity.** Randomized 8-wk-old LIS rats were immunized with two subcutaneous injections (1 wk apart) of  $\sim 10^8$  PFU of the individual viruses in a volume of 0.3–0.5 ml. In experiments with Ad5 and Ad12 viruses the control animals received an identical volume of PBS. In experiments with the recombinant adenoviruses, an equivalent volume of uninfected 293 cell extract was injected into control rats. In some experiments with the recombinant viruses, additional control animals were injected with 0.5 ml of PBS. 3 wk after the second injection the animals were challenged with various doses of the tumorigenic EcoC3 cells or Ad2/T13 cells inoculated subcutaneously. The animals were observed for at least 100 d for tumor development, and tumor size was measured with a caliper. This 50% tumor-producing dose (TPD<sub>50</sub>) was calculated where indicated by the method of Karber (16). At the end of the experiment all apparently tumor-free animals were killed, autopsied, and examined for the presence of tumors.

**Statistical methods.** The significance of differences in distribution of tumors between control and immunized rat populations was determined by the use of Fisher's exact test (17).

<sup>2</sup> Sawada, Y., M. Perricaudet, K. Raska, Jr., and T. E. Shenk. Manuscript submitted for publication.

TABLE I  
*Induction of Transplantation Immunity Against Adenovirus-transformed Cells With Wild Type Adenoviruses 5 and 12*

Immuniza- tion	Tumor-positive rats at Ad12-EcoC3 cell doses of:			TPD <sub>50</sub> *	Tumor-positive rats at Ad2-T13 cell doses of:			TPD <sub>50</sub> *
	10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>		10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>	
Control	—	5/5	2/5	1.3 × 10 <sup>5</sup>	—	4/4	1/5	2 × 10 <sup>5</sup>
Ad5	5/5	5/5	—	≤3.2 × 10 <sup>5</sup>	0/4	0/4	—	≥3.2 × 10 <sup>7</sup>
Ad12	0/5	0/5	—	≥3.2 × 10 <sup>7</sup>	4/4	4/4	—	≤3.2 × 10 <sup>5</sup>

Animals were injected subcutaneously with 10<sup>8</sup> PFU of the two viruses at 8 wk of age and boosted 1 wk later. Control animals were injected with PBS. 3 wk after the second injection, the animals were challenged subcutaneously with various doses of the two tumorigenic cell lines. The inoculated animals were followed for tumor development for 120 d.

\* The estimated dose producing tumors in 50% of animals, calculated by the method of Karber (16).

## Results

*Transplantation Immunity Induced by Wild Type Adenoviruses 5 and 12.* To study the adenovirus genes required for induction of specific TSTA immunity, we selected two syngeneic cell lines that are tumorigenic in competent adult rats. The EcoC3 cells were transformed with the cloned Eco RI-C fragment of Ad12 DNA; the Ad2/T13 cells were transformed with Ad2 virions.

All studies of adenovirus TSTA immunity reported so far used virus-transformed cells. The virus functions expressed in such cells usually have not been studied and it has not been demonstrated that effective transplantation immunity can be induced against cells transformed with the cloned DNA segment encoding the transforming region. For this reason the tumorigenicity and effects of immunization with wild type Ad5 and Ad12, respectively, were examined first. Both cell lines are highly tumorigenic in 12-wk-old rats, as can be ascertained from the TPD<sub>50</sub> values calculated for the subcutaneous inoculation (Table I).

Immunization with the two Ad serotypes offers a strong group-specific syngraft immunity which is apparent from the changed TPD<sub>50</sub> values in immunized animals. The two injections of Ad12 protect against tumor induction with EcoC3 cells, while Ad5 immunization protects against Ad2/T13 tumors. Growth of either tumor was not affected by immunization with the heterologous virus. This result confirms earlier studies that immunization with group A and group C adenoviruses, respectively, is not crossprotective (4, 5). It also shows that a strong syngraft immunity can be induced against EcoC3 cells transformed with the cloned fragment of the transforming region of Ad12 DNA, which do not express any additional Ad early functions. Strong transplantation immunity against Ad2/T13 cells was also induced with Ad2 immunization (not shown), in agreement with an earlier report (5). This result shows that examination of the role of the E1A and E1B transcription units in the protective effects of immunization with viable recombinant Ad5/Ad12 adenoviruses on the growth of EcoC3 and Ad2/T13 cells, respectively, is feasible.

*Induction of Syngraft Immunity Against EcoC3 Cells by Immunization With Recombinant Ad5/Ad12 Adenoviruses.* Three recombinant viruses were selected for studies of induction of TSTA immunity: *sub370-12E1AB*, *sub370-12E1A*, and

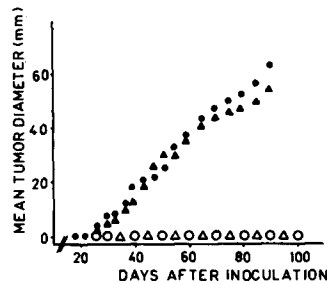


FIGURE 2. Growth of  $2 \times 10^6$  EcoC3 cells in syngeneic LIS rats immunized with different recombinant viruses. The LIS rats were immunized with 2 subcutaneous injections of  $10^8$  PFU of the recombinant adenoviruses. Control animals received two 0.5-ml injections of the lysate extract of uninfected 293 cells. 3 wk after the second injection the rats were challenged with  $2 \times 10^6$  EcoC3 cells. The average sizes of the tumors in each individual group of not less than three animals are shown. ●, control animals; ▲, *sub370-12E1B*, ○, *sub370-12E1A*; △, *sub370-12E1AB*.

*sub370-12E1B*.<sup>2</sup> As can be seen in Fig. 1, the entire E1 region of Ad5 was substituted with the Ad12 E1 region in the *sub370-12E1AB* virus. The E1A transcription unit only was substituted in the *sub370-12E1A* virus, and the E1B subregion was substituted in the *sub370-12E1B* virus. Immunization with these three viruses should establish which of the two subregions of the early region 1 is required for TSTA activity of Ad12.

In the first experiment the 8-wk-old rats were injected with two doses (1 wk apart) of the recombinant viruses ( $10^8$  PFU). Since the recombinant viruses were grown in the Ad5-transformed 293 cells, the control animals were injected twice with uninfected 293 extracts. 3 wk after the second injection the rats were challenged with  $2 \times 10^6$  EcoC3 cells. Results in Fig. 2 show growth of the EcoC3 tumors in rats immunized with the three viruses. While tumors grew in animals immunized with the *sub370-12E1B* virus, no tumors developed in rats injected with the *sub370-12E1AB* or *sub370-12E1A* viruses.

The protective effects of the immunization with *sub370-12E1AB* and *sub370-12E1A* virions were further tested with various doses of the inoculated tumorigenic EcoC3 cells (Fig. 3). Although the latency period was dependent on the inoculated cell dose, tumors in control animals developed with all doses used ( $2.5 \times 10^6$ – $2 \times 10^7$  cells). In rats immunized with *sub370-12E1AB* and *sub370-12E1A* viruses, respectively, no tumors developed even when animals were challenged with  $2 \times 10^7$  EcoC3 cells (Fig. 3D).

Results of four additional experiments on the effects of the immunization with recombinant Ad5/Ad12 adenoviruses on tumorigenicity of the EcoC3 cells are summarized in Table II.

It is apparent that immunization with *sub370-12E1AB* and *sub370-12E1A* viruses induces a strong syngraft immunity against EcoC3 cells. With the cell doses used, no animal immunized with these viruses developed tumors after subcutaneous inoculation challenge.

In animals immunized with the *sub370-12E1B* virus, the frequency of animals developing tumors (Table II) and kinetics of tumor growth (Fig. 2) were not different from those in control rats.

This result indicates that the TSTA immunity induced by adenovirus 12 is

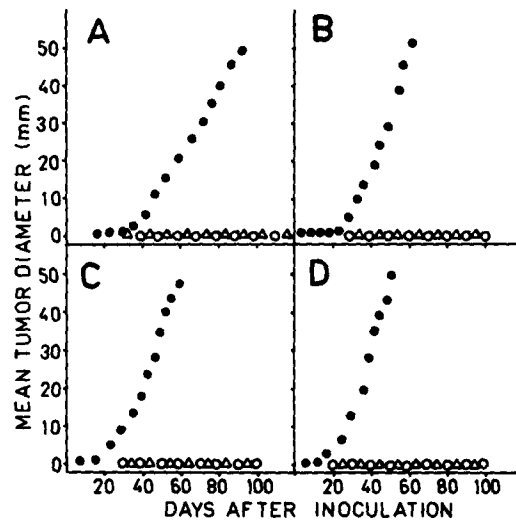


FIGURE 3. The EcoC3 tumors do not grow in animals immunized with viruses carrying the E1A transcription unit of Ad12. The LIS rats were injected twice with  $1.5 \times 10^8$  PFU of recombinant viruses (0.5 ml). The control rats received two 0.5-ml injections of the lysate of noninfected 293 cells. 3 wk after the second injection the rats were challenged with various doses of the EcoC3 cells: A,  $2.5 \times 10^6$ ; B,  $5 \times 10^6$ ; C,  $10^7$ ; D,  $2 \times 10^7$ . The growth of tumors was monitored in each group of not less than three animals: ●, control animals; ○, *sub370-12E1A*; △, *sub370-12E1AB*.

TABLE II  
*Induction of Transplantation Immunity Against Ad12-transformed Cells in Four Experiments With Ad5/Ad12 Recombinant Viruses*

Immunization	Exp. I $2 \times 10^6$ cells	Exp. II $2 \times 10^6$ cells	Exp. III $10^7$ cells	Exp. IV $2 \times 10^7$ cells
None	8/8	ND*	ND*	ND*
Control	6/6	5/5	3/3	3/3
<i>Sub370-12E1AB</i>	0/5	0/5	0/5	0/5
<i>Sub370-12E1A</i>	0/5	0/5	0/5	0/5
<i>Sub370-12E1B</i>	6/6	5/5	ND*	ND*

8-wk-old LIS rats were injected with  $\sim 1.3 \times 10^8$  PFU of recombinant adenoviruses and boosted 1 wk later. Control animals received two doses of lysate of the noninfected 293 cells. 3 wk after the second injection, the rats were challenged with indicated doses of the EcoC3 cells. The *in vitro* cell passage of the EcoC3 cells was as follows: Exp. I, passage 18; Exp. II, passage 21; Exp. III, passage 28; Exp. IV, passage 23. The individual animals were followed for tumor development for 100 d.

\* ND, not done.

dependent on the E1A region. Immunization with virions containing and expressing the E1 early region (*sub370-12E1AB*) or only the E1A Ad12 genes (*sub370-12E1A*) induces a strong protective immunity. The Ad12 E1B genes, however, induce no significant protection. Tumor induction was unaffected by immunization with *sub370-12E1B* virus.

*Effects of Immunization With Recombinant Ad5/Ad12 Adenoviruses on Tumorigenicity of Ad2/T13 Cells.* The conclusions drawn from the effects of immuni-

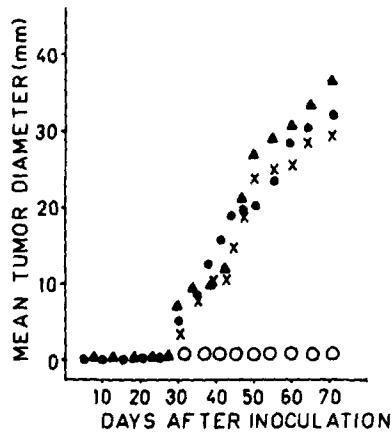


FIGURE 4. Growth of Ad2/T13 tumors in syngeneic rats immunized with recombinant adenoviruses. The 8-wk-old rats received two subcutaneous injections (1 wk apart) of  $1.3 \times 10^8$  PFU of the recombinant adenoviruses in 0.5 ml. The control rats were injected with 0.5 ml lysate extracts of uninfected 293 cells. 3 wk after the second injection the rats were inoculated subcutaneously with  $4 \times 10^6$  Ad2/T13 cells (passage 3) and were followed for tumor development. Mean tumor size in not less than three animals per group is shown: ▲, control rats; ●, rats immunized with the *sub370-12E1A* virus; ×, rats immunized with the *sub370-12E1AB* virus; ○, rats immunized with the *sub370-12E1B* virus.

zation with recombinant viruses on growth of the EcoC3 tumors could be further verified and expanded by a reciprocal experiment. If the conclusion that adenovirus TSTA activity is a function of the E1A genes is correct, immunization with *sub370-12E1AB* and *sub370-12E1A* virions should have no effect on growth of Ad2/T13 tumors. On the other hand, immunization with *sub370-12E1B* should confer syngraft immunity against cells transformed with a group C adenovirus.

The effects of immunization on growth of the Ad2/T13 tumors are shown in Fig. 4. It is apparent that tumor growth in animals immunized with *sub370-12E1AB* and *sub370-12E1A* viruses, respectively, is not different from that in rats immunized with the control 293 lysates. No tumors developed, however, in animals immunized with two doses of the *sub370-12E1B* virus. Results of three independent experiments using challenge with different cell dose are summarized in Table III. No protective immunity was seen in animals injected with virions containing the E1 region or the E1A subregion, respectively, of Ad12. On the other hand, no tumors developed in rats immunized with a recombinant virus *sub370-12E1B* containing and expressing the E1A region of Ad5. This result indicates that the Ad5 E1A genes and not the Ad5 E1B genes are responsible for induction of adenovirus group C TSTA.

### Discussion

It has been shown previously that adenoviruses can be subdivided into at least two groups according to their TSTA (18). It was demonstrated that immunization with Ad2 induced no protection against Ad12 tumors (4); later it was shown that the Ad12 (strain Huie) induces no immunity against group C adenovirus tumors (5). Analysis of TSTA induction by a series of Ad5 mutants in that study strongly

TABLE III  
*Induction of Transplantation Immunity Against  
 Adenovirus 2-transformed Cells in Three Experiments With  
 Ad5/Ad12 Recombinant Viruses*

Immunization	Exp. I 2 × 10 <sup>6</sup> cells	Exp. II 3 × 10 <sup>5</sup> cells	Exp. III 10 <sup>7</sup> cells
None	3/3	ND*	ND*
Control	3/4	3/3	3/3
<i>Sub370-12E1AB</i>	3/4	3/3	3/3
<i>Sub370-12E1A</i>	ND*	3/3	3/3
<i>Sub370-12E1B</i>	0/4	0/3	0/3

8-wk-old LIS rats were injected with  $\sim 1.3 \times 10^8$  PFU of the recombinant adenoviruses and boosted with the same dose 1 wk later. Control animals received two injections of lysate extracts of noninfected 293 cells. 3 wk after the second injection, the rats were inoculated with the indicated number of the Ad2/T13 cells. In vitro cell passage of the Ad2/T13 tumor cells in Exp. I was 7; in Exp. II and Exp. III, the cell passage was 3.

\* ND, not done.

suggested that the adenoviral TSTA is a function of the early region 1. It was not possible to determine, however, if a product of either E1A or E1B or of both regions is involved (5).

An ideal tool to clarify this point became available with the construction of viable recombinant Ad5/Ad12 viruses.<sup>2</sup> In the three viruses used in this study the early region 1 genes of Ad5 were substituted by the corresponding genes of Ad12 (Fig. 1). It has been demonstrated in earlier studies that these substituted regions are indeed expressed in the infected<sup>2</sup> and transformed cells (19).

In this study we first confirmed the TSTA activity of the Ad5 and Ad12 viruses. We also showed that a strong TSTA immunity can be produced against tumorigenic cells transformed with a cloned DNA fragment containing only the early region E1. These results provide compelling evidence that the adenoviral TSTA is a function of the E1A early region. This evidence is based on the effects of immunization on tumor induction with syngeneic Ad12- and Ad2-transformed tumorigenic cells. Viruses containing the E1A genes of Ad12 induce a strong TSTA immunity against EcoC3 cells expressing the E1 region of Ad12. The protective effects are not significantly affected by the origin of the E1B region in the virus used for immunization, whether from Ad12 or from Ad5. The *sub370-12E1B* virus containing only the E1B region of Ad12 has no protective effects on the growth of EcoC3 tumors. The virus containing Ad5 E1A genes, however, induces strong protection against tumors induced by Ad2/T13 cells. Tumorigenicity of Ad2/T13 cells was not affected by either the *sub370-12E1AB* or by the *sub370-12E1A* viruses.

In the earlier studies of effects of immunization with cells transformed with isolated fragments of Ad12 DNA, the Acc I-H fragment (0–4.7 map units) transformants failed to induce tumor immunity seen with cells transformed with the Hind III-G fragment (0–6.8 map units). The TSTA function was tentatively assigned to the left end of the E1B region (10). Analysis of a library of syngeneic cells transformed in vitro with the cloned fragments of the transforming region

revealed that cells transformed with Eco RI-C fragment (0–16.5 map units), Sal I-C fragment (0–10.3 map units), and Hind III-G fragment (0–6.8 map units) elicit cytolytic responses (11). Cells transformed with the cloned Acc I-H fragment (0–4.7 map units), however, failed to elicit cytolytic response and were not effectively lysed by CTLs or antibody raised against cells expressing both E1A and E1B regions. These results suggested that the Ad12-specific cell surface antigen is also a product of the left end of the E1B early region (11). This interpretation was supported by the demonstration that the 163 amino acid protein coded by this region is membrane associated and can be labeled by radioiodination of the cell surface (20). An analogous polypeptide coded by group C adenoviruses has also been localized in part within the plasma membrane (21). By definition, however, tumor-specific transplantation immunity can only be assessed *in vivo*. Data presented here indicate that the E1A rather than the E1B transcription unit controls adenoviral TSTA activity. The results of the transplantation immunity with both EcoC3 and Ad2/T13 cells are statistically highly significant. The Fisher's exact test analysis shows that the results with the EcoC3 cells and *sub370-12E1A* and *sub370-12E1AB* viruses are significant at a *P* value of  $6.29 \times 10^{-11}$ . Experiments with Ad2/T13 cells and the *sub370-12E1B* virus are significant at the level of  $P = 5.95 \times 10^{-5}$ . The TSTA may be coded by the E1A early region; no evidence has been provided as yet, however, that the E1A polypeptides are a part of cellular membrane. Alternatively, different antigens of cellular origin may be induced by the E1A genes of Ad5 as compared with Ad12.

The E1A region of different adenovirus serotypes has been shown to have multiple functions relating to tumorigenicity. It was shown to complement the *ras* genes for transformation (22), to selectively modulate expression of the class I MHC antigen in the transformed cells (23), to affect the cytoskeleton organization in infected cells (24), and to control sensitivity of the transformed cells to natural killer cells (19). Results presented here associate adenovirus E1A genes with an additional function: adenovirus tumor-specific transplantation antigen.

### Summary

Viable recombinant adenoviruses that carry a portion of the type 12 E1A and E1B transcription units in a type 5 background were used to identify genes controlling expression of the adenovirus tumor-specific transplantation antigen (TSTA). The TSTA immunity is not crossreacting between the group A and group C adenovirus serotypes. Viruses carrying the E1A region (*sub370-12E1A*), or both E1A and E1B (*sub370-12E1AB*) regions of Ad12, induce a strong transplantation immunity against tumors induced by syngeneic cells transformed with adenovirus 12, but fail to induce any protection against syngeneic cells transformed with adenovirus 2. Immunization with the virus carrying only the E1B region (*sub370-12E1B*) of adenovirus 12 induces no immunity to adenovirus 12 transformed cell line, but confers a strong protection against cells transformed with adenovirus 2. These results provide strong evidence that the adenovirus tumor-specific transplantation antigen is a function of the E1A early region.



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