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Reversal Effect of Thymus Grafts on Lepromatous Leprosy in Thymectomized-Irradiated Mice

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Leprosy microinfections are experimentally produced in the hind footpads of mice ([13, 21]), but histologically the lesions in the early stage do not closely resemble any of the disease forms seen in man. In the late infection, however, epithelioid cell granulomata are found—a feature of borderline leprosy—and dissemination to peripheral body sites occurs ([16]). Arrest or severely restricted multiplication of *Mycobacterium leprae* invariably occurs locally when a level of a few million bacilli is reached ("ceiling-level"), probably as a consequence of the host's immunologic response to this number of bacilli, though a histologically detectable lesion often remains for the lifespan of a mouse ([16]).

Thymectomy plus irradiation (900 r) in mice ablates immunologic responsiveness and *M. leprae* is able to multiply well beyond the limit normally imposed ([5, 14, 15, 22]). Footpad lesions are often palpable and simulate lepromatous (bacilliferous) leprosy with involvement of plantar and regional nerves. Curiously, such mice sometimes undergo a slow recovery to immune competence and show "burnt-out" leprosy. This kind of reversal also occurs invariably and much more quickly in association with inflammation, following injection of isogeneic lymphoid cells ([15]).

Restoration to immune competence is less than complete in thymectomized supralethally irradiated mice after injection of a large number of disassociated thymocytes ([11]), but after implantation of a thymus gland full immune competence is conferred upon the recipient's own lymphoid cells ([10]). Hence a preliminary investigation was carried out in order to study (1) the distribution of ^{51}Cr-labelled splenic or thymic mononuclear cells after injection into thymectomized-irradiated mice with lepromatous leprosy, and (2) the reversal effect a thymus graft has on an established lepromatous lesion in thymectomized-irradiated mice, with particular reference to leucocyte DNA synthesis.

MATERIALS AND METHODS

Immunosuppression of mice. Female CBA inbred mice (6 weeks old), 13–17 gm. body weight, were thymectomized and three weeks later exposed to 900 r whole body irradiation, (45 r per minute, ^{60}Co. source). Because the irradiation dose is supralethal, it was essential to give an intravenous injection of approximately 10^7 isogeneic bone marrow cells (in Hanks' balanced salt solution) in order to prevent death.

In irradiated recipients these marrow cells have a capacity of self-renewal and differentiation into clones of erythroid and myeloid cells ([24]) and it is assumed that few of the stem cells present are capable, in the absence of a thymus, of differentiating into immunologically competent lymphoid cells. Using the method of Larsen and Ainsworth ([9]), we calculated that about 3.3×10^3 of the marrow cells injected have a capacity to form clones in a recipient's spleen alone. Nonetheless, despite a massive cloning potential, few mice survive after injection of 10^6 bone marrow cells.

Leprosy infection. About three weeks after irradiation, 10^5 *M. leprae* (in 0.03 ml. of 1.0% albumin saline) were inoculated into the plantar aponeurosis of both hind footpads of the mice. A high proportion (53%) of bacilli were thought to be viable, using the criteria of Rees and Valentine ([17]), i.e., viable bacilli staining "solidly"

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with carbolfuchsin. The bacillary suspension was originally obtained by homogenization of a leproma excised from an untreated Malayan patient and had been passaged once in thymectomized-irradiated mice.

At intervals the footpads of the mice were palpated, and some mice were killed and had their footpads removed for bacteriologic or histologic investigation.

***In vitro* labelling of mononuclear leucocytes with ^{51}Cr.** Aseptic precautions were taken throughout. The spleens or thymuses were excised from exsanguinated mice (CBA females, 6-8 weeks old), minced with scissors, strained through a sieve into Hanks' solution chilled to 4°C, and passed through fine-mesh nylon to remove debris. The splenic cells were collected by centrifugation (500 g for 7 minutes) and erythrocytes were flash-lysed ([3]) by washing twice in Tris-buffered isotonic ammonium chloride solution (pH 7.2), and finally washed in Hanks' solution (4°C). Both cell types were suspended in medium TC 109 (*Difco*) plus 20 per cent decomplemented mouse serum (slightly acid pH) to which approximately 100 μc ^{51}Cr. (sodium chromate, *Radiochemical Centre, Amersham, England*) per 2.0×10^8 cells was added. Cells were labelled by incubation at 37°C for one hour and then washed four times in medium TC 109 (°C), and suspended in Hanks' solution ready for injection; 92 per cent of thymocytes and 77 per cent of splenic nucleated cells appeared viable by the dye exclusion test (1.0% eosin saline).

Thymectomized-irradiated lepromatous mice, and normal control mice of the same body weight, had either 5.0×10^7 thymocytes or 5.0×10^6 spleen cells (in 0.2 ml. Hanks' solution) injected into a tail vein about 7.5 months after infection. After four and 24 hours of injection of the cells groups of three to four mice were killed, and the putative number of ^{51}Cr-labelled cell which had reached the hind footpad, spleen and sciatic nerve, were estimated by the method of Bainbridge, Brent and Gowland ([2]). Counts were carried out on a Packard Autogamma Scintillation counter. Bainbridge *et al.* ([2]) had demonstrated that the majority of soluble ^{51}Cr-sodium is eliminated from the body within 1.5 hours of injection.

Thymus transplantation. The thymus was carefully removed from a newborn CBA mouse and immediately transferred under the left kidney capsule of a thymectomized-irradiated mouse, using ether anesthesia, at about eight months after inoculation of *M. leprae*. A group of 12 mice were selected from the pool available, which showed slight swelling of infected footpads. Footpads were then palpated regularly and the amount of swelling elicited by the graft was recorded arbitrarily. A group of similar mice, without a thymus transplant, were set aside as controls.

Lepromin testing. About 2.5 months after thymus grafting, recipients and control mice had 0.03 ml. of standard Mitsuda-type lepromin injected into the right ear lobe (groups of 6 mice).

Autoradiographic techniques for ^{3}H-thymidine labelled leucocytes. At the height of the inflammatory reaction, following implantation of a thymus, mice were injected intravenously with 0.75 μc ^{3}H-thymidine (*Radiochemical Centre*) per gm. body weight. Control lepromatous mice were similarly injected. After four hours the mice were anesthetized with ether and one hind footpad was biopsied; after a further 20 hours the mice were killed and the contralateral footpad was removed. Briefly, the footpads were fixed in Carnoy's solution, embedded in paraffin-wax, sectioned at 4 μ thickness, placed on glass microscope slides, and coated with G5 liquid photographic emulsion (*Ilford*). The slides were exposed for four weeks (−6°C) and developed in ID 19 (*Ilford*) according to the method of Rogers ([19]). Bacilli were stained through the emulsion with New fuchsin ([7]) and tissues were counterstained with Harris-hematoxylin.

RESULTS

Thymectomized-irradiated mice all showed a markedly enhanced susceptibility to infection with *M. leprae*; gradual enlargement of the infected paws occurred,

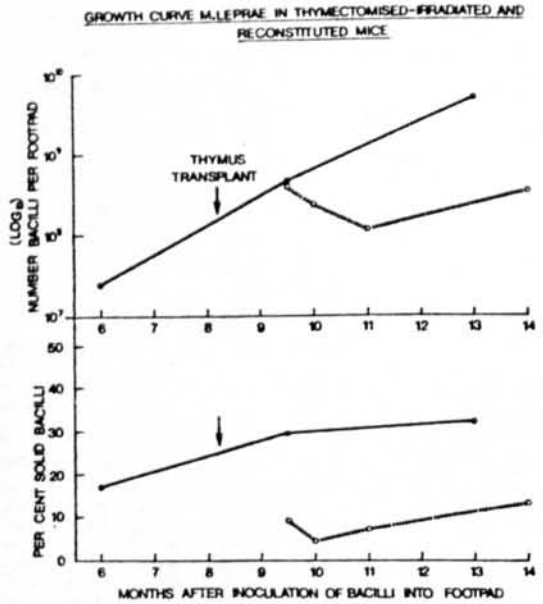

FIG. 1. Growth curve of *M. leprae* in the footpads of thymectomized-irradiated mice (●——●), and thymus-reconstituted mice (o ..... o), showing arrest of bacillary multiplication and rapid degeneration of bacilli following thymus graft. Each point represents the mean footpad content of bacilli from two (4 footpads) to five (8 footpads) mice.

beginning about 7.5 months after inoculation of bacilli (Fig. 1).

Distribution of ^{51}Cr-labelled splenic and thymic cells in lepromatous lesions. At the time of injection of radio-labelled cells into lepromatous mice the yield from a footpad was about 10^8 bacilli (20% viable), but the lesions present were only just palpable. Because of relatively poor retention of the radio-label by cells which had been injected (2) the period of measurement of radioactivity in the various tissues examined (the infected paws, sciatic nerve and spleen) was confined to within 24 hours. Nevertheless this was considered an adequate time for the labelled cells in the bloodstream to reach and infiltrate lesions. Results summarized in Tables 1 and 2 show that neither splenic nor thymic cells had a predilection for lepromatous lesions.

Elicitation of erythematous inflammation in lepromatous lesions by thymus grafts. Development of a neonatal thymus gland transplanted into a thymectomized-irradiated mouse took about four weeks, as judged histologically. After four and a half to six weeks of implantation of the thymus, massive erythematous inflammation suddenly occurred in infected paws. This swelling then slowly regressed during the next seven weeks. Inflammation was concurrent with rapid destruction of bacilli (Fig. 1), the morphologic index falling from 24–29 per cent to 3–14 per cent within eight weeks of thymus grafting. Thus the reaction elicited undoubtedly caused reversal

TABLE 1. *Distribution of Cr^{51}-labelled spleen cells (5.0 × 10^6/mouse) in lepromatous and normal mice (groups 3–4 mice).*

Time	Putative number of spleen cells (× 10^3) recovered (& percentage) ± S.D.		
	Footpad[a]	Spleen	Sciatic nerve[a]
Lepromatous mice			
4 hours	4.79 ± 0.63 (0.09 ± 0.01%)	956.3 ± 125.0 (19.11 ± 0.77%)	1.24 ± 0.27 (0.02%)
24 hours	4.76 ± 1.38 (0.08%)	860.7 ± 76.0 (17.6 ± 1.62%)	0.33 ± 0.20 (0.01%)
Control mice			
4 hours	11.69[b] (0.24%)	1,018.1 ± 132.0 (20.43 ± 0.88%)	1.84 ± 0.50 (0.07%)
24 hours	9.32 ± 0.85 (0.18 ± 0.01%)	869.2 ± 253.0 (17.8 ± 6.4%)	0.66 ± 0.26 (0.01%)

[a] Site of *M. leprae* infection, both hind footpads and sciatic nerves included.
[b] Two mice only.

TABLE 2. *Distribution of Cr^{51}-labelled thymocytes (5.0×10^7 cells/mouse) in lepromatous and normal mice (groups 3–4 mice).*

Time	Putative number of thymocytes (& percentage) recovered $\times 10^3$ (± S.D.)		
	Footpad[a]	Spleen	Sciatic nerve[a]
Lepromatous mice			
4 hours	402.6 ± 114.5 (0.84 ± 0.11%)	12,320.0 ± 511.0 (24.6 ± 3.2%)	6.03 ± 2.8 (0.18 ± 0.56%)
24 hours	246.7 ± 25.1 (0.58 ± 0.55%)	12,362.2 ± 343.0 (24.8 ± 0.7%)	2.06 ± 0.6 (0.04 ± 0.01%)
Control mice			
4 hours	151.6[b] (0.3%)	22,166.7 ± 317.0 (44.3 ± 5.0%)	9.4 ± 6.4 (0.28 ± 0.11%)
24 hours	128.0 ± 11.9 (0.26 ± 0.05%)	13,004.0 ± 289.5 (26.0 ± 2.3%)	1.62 ± 0.6 (0.04 ± 0.01%)

[a] Site of infection with *M. leprae*, both hind footpads and sciatic nerves included.
[b] Two mice only.

of the lepromatous condition.

Lepromin testing in the ear lobe of reconstituted mice showed a response only detectable histologically (Fig. 2), which was consistent with a positive Mitsuda reaction, but the response could have been evoked by a disseminated lesion already established in the ear lobe when the thymus was transplanted.

Histologically, the footpad reaction resembled a cell-mediated immune reaction rather than an Arthus reaction; there were few polymorphonuclear leucocytes, some edema, and vast accumulation of lymphocytes, in all infected tissues. Globi, both in macrophages and to a lesser extent in striated muscle fibers, underwent disintegration and bacilli were nearly all degenerate (Fig. 3). In fact, by seven months after thymus transplantation bacillated muscle

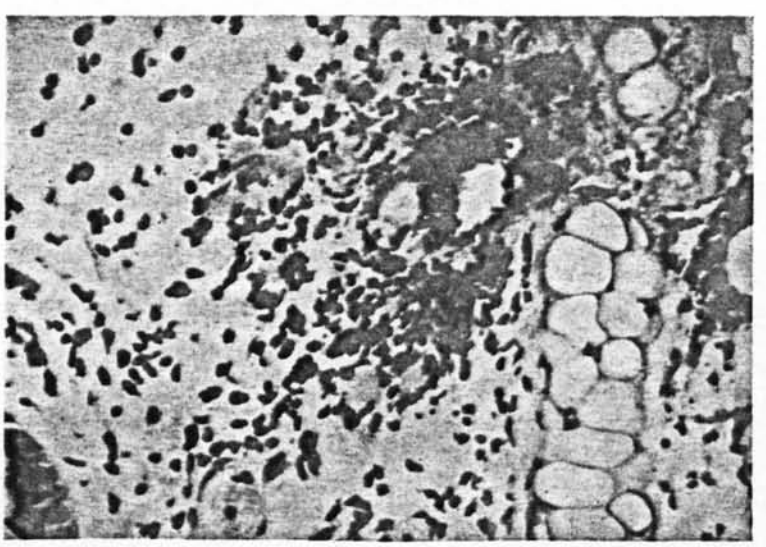

FIG. 2. Characteristic cellular response to lepromin in the ear lobe of a thymus-reconstituted mouse. X180.

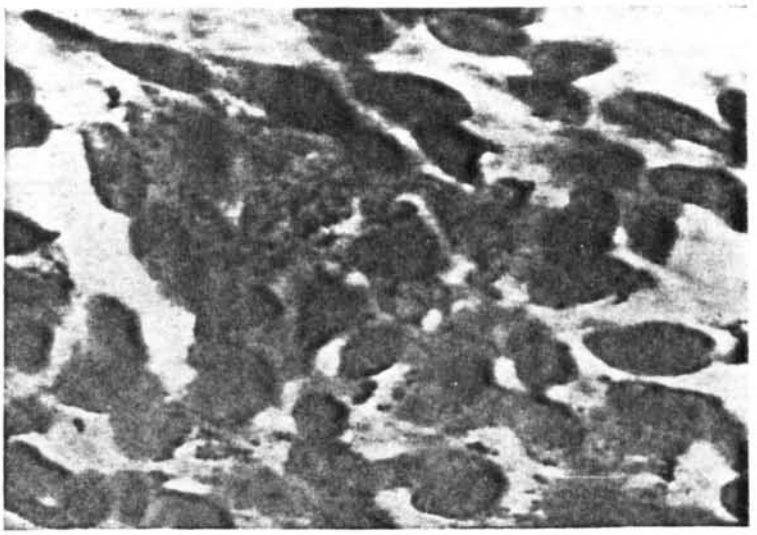

FIG. 3. Footpad section seven months after thymus reconstitution showing complete replacement of a muscle area following destruction of muscle fibers, with an epithelioid cell granuloma. The bacilli appear degenerate. X900.

fibers had been invaded by inflammatory cells and replaced largely by an epithelioid cell granuloma. However, by this time even some control mice had begun to show inflammation, and the now macroscopic lesion had upgraded to a borderline condition with epithelioid cell granulomata involving all tissues (Fig. 4).

Reactional changes due to immune reconstitution were less severe in both plantar and sciatic nerves. Nonetheless, when lymphocytes infiltrated bacillated nerves the bacilli underwent degeneration. This was more common after thymus grafting.

Incorporation of ^{3}H-thymidine into lymphocytes in lepromatous lesions during reversal induced by a thymus graft. Division of at least some lymphocytes is evoked by antigens. A period of DNA synthesis

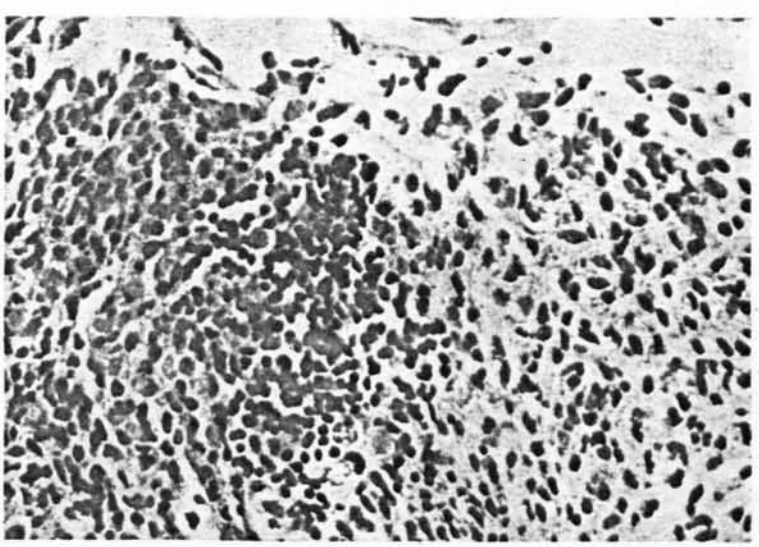

FIG. 4. Footpad section of a control mouse, 14 months after infection, showing spontaneous reversal with epithelioid cell granuloma involving all tissues. X210.

precedes mitosis and the cell enlarges to form the immunoblast. Cell division can therefore be demonstrated autoradiographically following uptake of ^{3}H-thymidine by DNA. Accordingly a daughter cell shares half-as-much thymidine in its nucleus as the progenitor cell. Hence the identification of transformed cells is made possible by silver grain counts in the autoradiograph. However, it is not certain whether all lymphocytes that incorporate ^{3}H-thymidine and form immunoblasts divide.

At the height of the inflammatory reaction following reconstitution, a single pulse injection of ^{3}H-thymidine revealed that surprisingly few cells became labelled (approximately 0.2%). By four hours after injection of the radio-label the vast majority of labelled cells were large immunoblasts, whereas after a further 20 hours a 40-60 per cent reduction in grain count occurred (approximately 200 labelled cells counted) and the majority of labelled cells appeared to be normal lymphocytes. Hence the period of cell mitosis was probably less than 24 hours. In control mice far fewer cells incorporated ^{3}H-thymidine.

Only rarely was a labelled macrophage seen, but most macrophages were heavily parasitized and had probably lost the ability to divide. Polymorphonuclear leucocytes failed to incorporate ^{3}H-thymidine, but it is well known that these cells do not divide after their maturation in hemopoietic tissues. Some fibroblasts, particularly those in association with the degenerating median plantar nerve, became labelled.

DISCUSSION

According to Ridley ([18]) reversal reactions in leprosy are associated with an upgrading of immunity and occur in lesions of borderline or near-lepromatous patients during treatment, resulting in a diminution of the bacterial load. Reversal in lepromatous mice, therefore, is unique, for patients with lepromatous leprosy probably do not show this phenomenon.

Susceptibility to lepromatous infection in mice is dependent on a powerful and long-term suppression of cell-mediated immunity ([5, 14, 15, 22]). Nonetheless, even in thymectomized-irradiated (900 r) lepromatous mice there was a slow, but probably partial, recovery to immune competence, greatly potentiated by a thymus transplant, which resulted in a borderline condition. Incorporation of ^{3}H-thymidine by lymphocytes in leprosy lesions undergoing thymus-induced reversal, or slow upgrading of immunity, was seen to occur only in a small minority of the cells forming the granuloma. This suggests that many cells which contribute toward leprosy granuloma formation infiltrate from the bloodstream and undergo only occasional division within the lesion. In immuno-suppressed mice the extremely slow spontaneous return to immune competence was probably due to the presence of a few immunologically competent cells in the bone marrow given after supra-lethal irradiation. The influence of the thymus graft, perhaps in connection with bone marrow cells, seemed necessary to potentiate the reversal reaction.

Neither splenic nor thymic mononuclear cells labelled with ^{51}Cr-sodium showed a predilection for lepromatous lesions after their injection into the bloodstream. However, such donor lymphocytes, particularly those gathered from the spleen, would be given the opportunity *in vivo* to become specifically sensitized to *M. leprae* antigens, and so should eventually produce a cell-mediated inflammatory response. This might attract nonsensitized lymphocytes into the lesion. Lymphoid cell replacement in thymectomized-irradiated lepromatous mice has shown that this is the likely sequence of events ([15]). Unlike spleen cells, thymocytes would probably not become sensitized to *M. leprae* antigens, but a proportion might cooperate with certain bone marrow-derived lymphocytes, which would then be made reactive to antigen ([20, 23]). Injection of lymphocytes which have been sensitized to *M. leprae* antigens, i.e., "adoptive-transfer" of immunity, should elicit a greater attraction for leprosy lesions. If so, this would provide an indirect method, perhaps the only method, for testing various vaccines against *M. leprae* infection in immuno-suppressed mice. If a comparison can be made with rats, small

lymphocytes, which have a short life-span in blood, were the only cells from thoracic duct lymph which have been demonstrated to accumulate in tissues with non-specifically induced inflammation (8). These findings suggest that the reversal reaction in leprosy may require this special category of lymphocytes which are probably instigators of cell-mediated immunity.

Depression of cell-mediated immunity in lepromatous patients is well known and was found by Turk and Waters (25) to be associated with a replacement of paracortical areas (or thymic-dependent areas) of lymph nodes with reticulo-histiocytes. The histologic appearance of lymph nodes resembled that seen in animals administered antilymphocytic serum, which also depresses cell-mediated immunity and enhances leprosy infection in thymectomized mice (6). Mice infected with *Mycobacterium lepraemurium* ("rat leprosy bacillus") showed similar depression of immunity and similar histologic changes in lymph nodes (12). Furthermore, in the mouse thymus there was progressive depletion of cortical thymocytes, and ultimately complete replacement by parasitized macrophages occurred. The defect in cell-mediated immune responses is thought to be secondary to the infection. The thymus gland, which is essential for the maturation of certain lymphoid cells to immune competence, is most probably involuted, or vestigial as a result of ageing, and unable to be repopulated in subjects with lepromatous leprosy. It is possible that a thymus graft would benefit lepromatous patients by bringing about some degree of immune reconstitution. Unfortunately, if indeed a thymus graft would mature in the patient, there is a possible danger of evoking a severe leprosy reaction. To date, implantation of a thymus homograft fragment has been used successfully in infants with DiGeorge's syndrome (4), or thymic aplasia (1), without causing a graft-versus-host reaction.

SUMMARY

Reversal reaction is associated with an upgrading of immunity in borderline or near-lepromatous lesions in man with diminution of the bacterial load.

Lepromatous leprosy is produced in thymectomized-irradiated (900 r) mice. In this investigation, however, lepromatous leprosy similarly produced showed a slow upgrading of cellular immunity in the late infection, resulting in a borderline condition. Recovery of immune competence is due to either the presence of a small number of immune-competent lymphocytes in the suspension of bone marrow cells, which are injected immediately following irradiation (essentially to prevent death), or to the fact that not all lymphocytes in the body are destroyed following exposure to 900 r.

Implantation of an isogeneic thymus (neonatal donor) beneath the kidney capsule significantly potentiated reversal; after about five weeks a massive, though transient, erythematous inflammation occurred in infected paws associated with a rapid destruction of *M. leprae*, although microcolonies of bacilli within striated muscle fibers were somewhat less affected. Histologically the lesions simulated a cell-mediated rather than an Arthus reaction, though some polymorphonuclear leucocytes were evident. Viable splenic or thymic ^{51}Cr-labelled mononuclear cells injected intravenously into lepromatous mice did not show a predilection for lesions. Nonetheless, labelled cells localized at random in infected and uninfected paws. Moreover, there was little mitotic activity of the cells (incorporation of ^{3}H-thymidine into DNA) constituting the lesions at the height of inflammatory reactivity in thymus-reconstituted mice.

These findings suggest that a reversal reaction requires a special category of lymphocytes—probably small lymphocytes, instigators of cell-mediated immunity. These small lymphocytes are found preponderately in lymph and to a lesser extent in peripheral blood, from which they reach leprosy lesions, especially if specifically sensitized.

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