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The synthetic, oxidized C-terminal fragment of the *Plasmodium berghei* circumsporozoite protein elicits a high protective response

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A polypeptide of 69 amino acids (PbCS 242–310) encompassing the C-terminal region of the circumsporozoite protein of *Plasmodium berghei* (PbCS) was generated using solid-phase peptide synthesis. The immunological and protective properties of peptide PbCS 242–310 were studied in BALB/c mice (H-2^d). Two subcutaneous injections, in the presence of IFA at the base of the tail, generated (i) high titers of anti-peptide antibodies which also recognized the native *P. berghei* CS protein, (ii) cytolytic T cells specific for the K^d-restricted peptide PbCS 245–253 and (iii) partial CD8$^+$-dependent protection against sporozoite-induced malaria. The same frequencies of peptide PbCS 245–253 specific CD8$^+$ T cells were found by IFN-γ ELISPOT in the draining lymph nodes of animals immunized with the short optimal CTL peptide 245–253 or with the polypeptide 242–310, indicating that the longer polypeptide can be processed and presented *in vivo* in the context of MHC class I as efficiently as the short CTL peptide. Interestingly, higher levels of IFN-γ producing CD8$^+$ T cells and protection were observed when the four cysteine residues present in the C-terminal peptide were fully oxidized. These findings underline the potential importance of the chemical nature of the C-terminal fragment on the activation of the immune system and concomitant protection.

Key words: Vaccination / Peptide / CTL / Parasite immunity / Parasite

1 Introduction

Malaria is a parasitic disease transmitted during the blood meal of infected mosquitoes which inoculate sporozoites into the mammalian host. Within minutes, sporozoites invade hepatocytes and develop into merozoites intracellularly by asexual schizogony. The merozoites then invade cyclically red blood cells, thereby inducing the appearance of the typical malaria symptoms. The life-cycle is completed when gametocytes are ingested during the blood meal of the mosquito vectors.

Protective immunity against malaria can be obtained by immunizing mice and humans with irradiation-attenuated sporozoites [1]. This immunity is the result of the effect of neutralizing antibodies recognizing free sporozoites in the blood stream and of CD4$^+$ and CD8$^+$ T cells which prevent the development of the parasite hepatic forms [2]. One aim in malaria vaccine research is to mimick the protective immune response induced by injection of irradiated sporozoites.

Murine malaria models have been extensively studied in order to characterize the immune mechanisms required to eliminate malaria hepatic stages. CD4$^+$ and CD8$^+$ T lymphocytes were shown to be involved in the protection mechanism at the liver stage. CD4$^+$- dependent protection was acquired through injection of T cell clones obtained after immunization with irradiated sporozoites [3] or after injection of synthetic peptides covering epitopes in the N-flanking region of the CS of *P. berghei* [4]. Similarly, immunization with a linear synthetic peptide from the sporozoite surface protein 2 (SSP2) of *P. yoelii* also provided CD4$^+$-and IFN-γ -dependent protection in mice [5].

CD8$^+$-dependent protection was also reported using various immunization protocols. Protection induced by irradiated sporozoites was absent upon depletion of CD8$^+$ T cells [6] or in MHC class I knockout mice [7]. Furthermore, injection of CS protein-specific CTL clones [8, 9]

Abbreviation: PbCS: *Plasmodium berghei* circumsporozoite protein

conferred full protection against *P. berghei*. Studies using Fas, Fas L, perforin and IFN-γ receptor-deficient mice indicated that protection is mediated by soluble factors like IFN-γ and TNF-α [10, 11].

Most of the studies undertaken so far have concentrated on the use of peptides stimulating one or the other facet of the immune system. In the design of a malaria vaccine exploitation of multiple immune mechanisms against parasite infection should be achieved and this might provide the desired level of protection against sporozoite challenge.

We have already shown that long polypeptides containing N- or C-terminal parts of *P. falciparum* CS protein induced neutralizing antibody responses in mice which prevented *P. falciparum* sporozoite penetration *in vitro* [12]. These peptides are also recognized by sera or PBL of people living in endemic areas [13–15]. Moreover, long polypeptides generate $CD8^+$ T cell responses *in vivo*, indicating that they can be adequately processed and presented in the context of MHC class I molecules *in vivo* [16, 17]. Here, we extend these studies to a model where *in vivo* protection can be assessed and we confirm that injection of polypeptide PbCS 242–310 induces a broad stimulation of the immune system. Indeed, peptide specific $CD8^+$ T cells, antibodies specific for the native protein, T helper cell proliferation and $CD8^+$-dependent protection against sporozoite-induced infection were detected. Importantly, oxidation of the four cysteine residues present in the C-terminal fragment induced a higher number of IFN-γ producing lymphocytes and a higher degree of protection against a parasite challenge.

2 Results

2.1 Immunological response to the fully reduced PbCS 242–310 C-terminal peptide

BALB/c mice were immunized twice with peptide PbCS 242–310 in IFA. The presence of peptide-specific antibodies was assessed 7–10 days after the second injection. A high titer of peptide-specific antibodies (1:300,000) was detected by ELISA and cross-reactivity with the native CS protein (1:25,000) was demonstrated by IFAT on *P. berghei* air-dried sporozoites. Recognition of sporozoites was specific since it was inhibited by the addition of competitor PbCS 242–310 peptide.

To assess the CTL response, spleen and LN immune cells were restimulated *in vitro* with the well known CTL epitope PbCS 245–253 [18], peptide PbCS 242–310 and overlapping peptides B11-B17 covering the entire C-terminal sequence. High levels of cytotoxicity were detected in spleens and LN of immunized mice restimulated with peptides 245–253 and 243–262, whereas lower levels were induced by peptides 233–252 and 242–310 (data not shown). The cytotoxic activity was similar to that obtained in mice immunized with the optimal nonameric peptide PbCS 245–253 as determined by CTL activity (Fig. 1) or by the number of IFN-γ spot forming cells (Fig. 2A). The K^d restriction of the CTL response was confirmed using transfected L cells expressing K^d, D^d or L^d MHC class I molecules (data not shown).

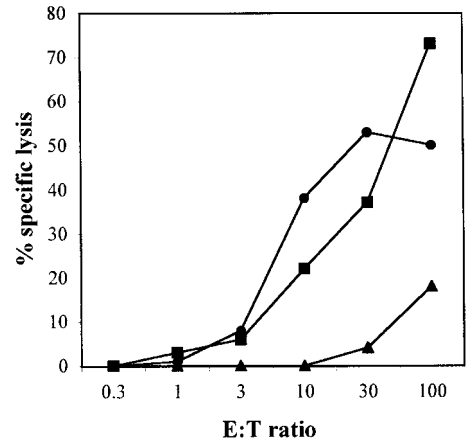

Fig. 1. Evaluation of the cytotoxic activity in mice immunized with peptide PbCS 245–253 or PbCS 242–310. BALB/c mice were immunized twice s.c. with 20 μg of the reduced peptide PbCS 242–310 (●) or 4 μg of PbCS 245–253 and 100 μg of P30 (■) in IFA. Ten days after the boost, spleen cells were isolated and cultured for 1 week in the presence of PbCS 245–253 pulsed P815 cells. A group of mice immunized with IFA alone was evaluated and served as negative control (▲). The results shown represent, for each E:T ratio, the difference of the percentage of lysis between the peptide PbCS 245–253 pulsed and unpulsed target cells.

2.2 Immunization with PbCS 242–310 peptide in IFA confers $CD8^+$-dependent protection to BALB/c mice exposed to parasite-bearing mosquitoes

BALB/c mice immunized twice s.c. at the base of the tail with 20 μg PbCS 242–310 peptide in IFA were submitted to a parasite challenge 7–10 days after the peptide boost. An important level of specific protection was obtained in the mice immunized with the PbCS 242–310 (Table 1, Experiment A). To characterize the mechanisms of protection, T cell depletion was performed *in vivo*. Clearly, the depletion of $CD4^+$ T cells did not significantly modify the observed protection. In contrast, depletion of $CD8^+$ T cells by isotype-matched $CD8^+$-specific antibodies prevented peptide-induced protection *in vivo*, since a baseline level of protection was observed (Table 1, Experiment A).

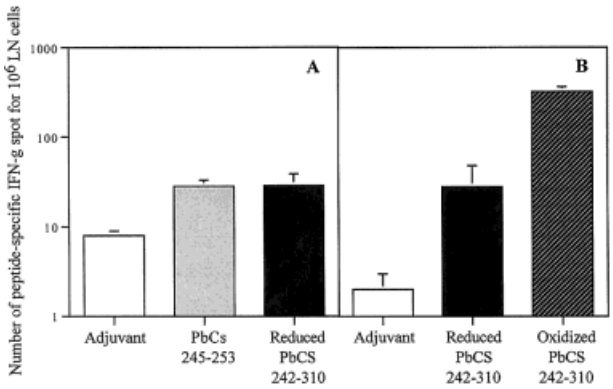

Fig. 2. Determination of peptide PbCS 245–253 specific T cells by IFN-γ ELISPOT in LN cells. Mice were immunized with various antigen preparations in IFA at the base of the tail on day 0 and 3–4 weeks later: (A) peptide PbCS 242–310 or peptides P30/PbCS 245–253; (B) reduced or oxidized PbCS 242–310. Inguinal and periaortic draining lymph nodes of two mice per group were removed 10–20 days after the second injection and cells obtained were pooled. The presence of specific T cells was assessed directly by ELISPOT in the presence of irradiated P815 pulsed or not with peptide PbCS 245–253. Mice immunized with IFA only were used as controls. Results are representative of two (A) and three (B) experiments and are expressed as the difference of spots obtained when cells were incubated with P815 cells pulsed or not with the PbCS peptide 245–252.

2.3 Immunological properties of the oxidized PbCS 242–310 C-terminal peptide

Since the four cysteines present in the C-terminus of the native CS protein are likely to be oxidized [19, 20], the fully reduced C-terminal peptide was allowed to undergo full oxidation by air-exposing it in an aqueous solution at pH 8.0 for 10 days at room temperature. The immunological properties of this preparation were then compared with the fully reduced material in terms of antibody production, T cell proliferation, CTL response and protective capacity. While no substantial difference was obtained in antibody titers and T cell proliferation (data not shown), the number of IFN-γ ELISPOT obtained with the oxidized material in three different experiments was consistently greater than that observed for the fully reduced peptide (Fig. 2B). Experiments performed with purified LN and spleen cells indicated that the response obtained in the ELISPOT assay was confined to the $CD8^+$ T cell population (Fig. 3). Similarly, the degree of protection obtained with the oxidized material was consistently higher than that observed with the reduced molecule (Table 1, Experiments B, C).

3 Discussion

Our present and previous results [16, 17] demonstrate that long polypeptides are efficient immunogens. Here, immunization with peptide PbCS 242–310 not only generates a wide spectrum of immune responses but also provides $CD8^+$-dependent protection against

Table 1. Protection studies in BALB/c mice using reduced or oxidized peptide PbCS 242–310[a)]

Experiment	Immunization	Treatment	Protected/Exposed	% Protection
A	IFA		2/9	22
	Reduced PbCS 242–310	Anti CD4	12/20	60
	Reduced PbCS 242–310	Anti CD8	3/20	15
	Reduced PbCS 242–310		12/20	60
B	IFA		1/7	14
	Oxidized PbCS 242–310		6/7	84
C	IFA		2/8	25
	Reduced PbCS 242–310		4/8	50
	Oxidized PbCS 242–310		8/8	100

a) BALB/c mice were immunized twice with the indicated preparations. Anti-CD4 and -CD8 treatment was performed prior to sporozoite injection as described in Sect. 4. Parasitemia was determined by Giemsa stained bloodsmears from day 5 to day 14. Mice were considered protected when no parasites were detected on day 14. The data shown in experiment A represent the accumulated results of two individual experiments.

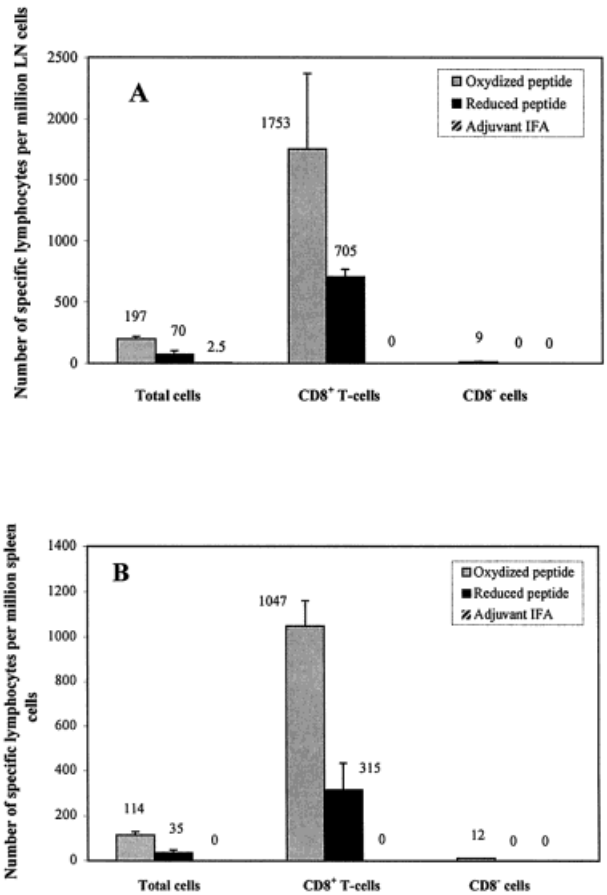

Fig. 3. CD8$^+$ T cell dependence of peptide PbCS 245–253 specific T cells in LN and spleen cells. *Ex vivo* PbCS 245–253 peptide-specific IFN-γ ELISPOT assays were performed 10 days after the second injection. BALB/c mice were immunized two times s.c. at 3-week interval with 20 μg of oxidized (gray column) or reduced (black column) peptide PbCS 242–310 in IFA. A group of mice immunized with adjuvant alone is represented in the dashed column. Inguinal and periaortic LN (panel A) and spleen (panel B) cells from two individual mice were plated prior or after cell treatment with anti-CD8a antibody: left columns, no treatment; center columns, CD8$^+$ T cells; right colums, CD8$^-$ cell population. Results are expressed as the difference of spots obtained when cells were incubated with P815 cells pulsed or not with the PbCS peptide 245–252.

sporozoite-induced infection. In particular and unexpectedly, the oxidized material induces a higher number of IFN-γ-producing T cells and a better degree of protection against a sporozoite challenge. At this stage, we can only speculate on the mechanism by which a better IFN-γ-producing CD8$^+$ T cell response is elicited. From *in vitro* studies, we have observed that the oxidized form is more resistant to proteolytic degradation than the reduced material. It is, therefore, possible that this property might lead to a longer half-life of the peptide *in vivo*. Alternatively, the oxidized material might interact more efficiently with specific cell surface receptors specific for the C-terminal part of the CS protein [19]. Either or both mechanisms may provide for a more efficient antigen presentation to specific CD8$^+$ T cells.

We had previously reported that immunization of BALB/c mice with a combination of tetanus toxin-derived helper peptide P30 and CS-derived peptide PbCS 245–253 confers protection only when immune cells were transferred to naive recipients [21]. In the present study, the injection of a reduced or oxidized long synthetic polypeptide covering the C-terminal region of the CS protein conferred protection against *Plasmodium berghei*. While the number of IFN-γ–producing T cells detected was higher in mice immunized with the oxidized polypeptide, it has to be noted that the injection of the reduced form induced a response which is similar to the response generated by the injection of the optimal nonameric peptide PbCS 245–253. However, animals which have been immunized with the reduced peptide were partially protected against a sporozoite challenge in a CD8$^+$-dependent manner. These results therefore suggest that not only the amplitude of the CD8$^+$-response is important but also that the generation of a multi-facet immune response can partially compensate a less important CD8$^+$ response.

It has been widely and independently demonstrated that T helper cells and antibodies can independently provide protection against malaria exoerythrocytic stages [3, 4, 22–24]. Here, we have shown that the peptide-specific antibodies also have the capability to recognize the native protein on *P. berghei* sporozoites and, therefore, it is likely that they can neutralize sporozoites *in vivo*. This might lead to a reduction in the number of infected hepatocytes and to protection mediated by CD8$^+$ T cells as a result of the modification of the balance between the hepatic stages and CD8$^+$ T cells in favor of the latter. In addition, injection of peptides PbCS 242–310 also induced Th1 cell proliferation. In this study, however, protection does not seem to be dependent on CD4$^+$ T cells. Here, CD4$^+$ T cells may play a crucial role in the initiation of a peptide-specific immune response by providing help to specific CTL or B cells. However, it can not be excluded that after anti CD4 antibody treatment the remaining T cells (about 5%) may eliminate a certain proportion of malaria liver stages by secretion of IFN-γ or by a yet unknown mechanism [10].

The data obtained in this animal study were the foundation for using the oxidized CS terminal fragment of *P. falciparum* in an on-going Phase I human trial. The result so far obtained indicate that antibody, T cell proliferation

and CTL responses are also obtained in humans (manuscript submitted).

Although the exact mechanisms leading to the generation of a more substantial $CD8^+$ response *in vivo* remain unclear, our studies show the importance of structural parameters on the elicitation of the immune response and suggest that the concertation of sub-optimal multiple immune responses can lead to protection against a sporozoite challenge.

4 Materials and methods

4.1 Peptide synthesis and analysis

All the peptides used in this study were chemically synthesized using solid-phase F-moc chemistry, as described by Merrifield and Atherton [25]. Chemicals and solvents used for the synthesis were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland), Novabiochem (Läufelfingen, Switzerland) and Fluka (Buchs, Switzerland). The 9-mer peptide PbCS 245–253 corresponds to an identified CTL epitope [8]. Polypeptide PbCS 242–310 covering the C-terminal region of the circumsporozoite protein of *Plasmodium berghei* ANKA strain [26] was obtained as previously described in detail for the *Plasmodium falciparum* analogue [12]. Briefly, the polypeptide was prepared on a p-alkoxybenzylalcohol resin (Wang resin) with a degree of substitution of 0.4 mmol/g. A 10-fold excess of F-moc amino acid derivatives and a 30 min coupling time were used. The crude polypeptide was purified by a combination of size exclusion chromatography (Sephadex G25, Pharmacia, Sweden) and RP-HPLC (W-Porex 5 C4, 250 × 10 mm, Phenomenex, Rancho Palos Verdes, USA) using a 10–50% CH_3CN gradient in 0.1% TFA/H_2O in 40 min with a flow rate of 3 ml/min. The 20-mer peptides B11-B17 which overlap by 10 residues and encompass the sequence PbCS 233–312 were purified by size exclusion chromatography. The degree of purity of all peptides was analyzed by RP-HPLC (C18 analytical column). Amino acid composition of purified peptides was determined according to Knecht [27] and the molecular weight was confirmed by mass spectrometry on an LDI 1700 Mass Monitor (Linear Scientific Inc., Reno, NV, USA) or a Voyager-DE (PerSeptive Byosystem, Framingham, MA, USA).

4.2 Reduction and oxidization of PbCS 242–310

The reduced form of PbCS 242–310 was obtained by treating the HPLC purified polypeptide with a 100 molar excess of DTT for 36 h at 37° C. After elimination of the DTT excess by size exclusion chromatography, a portion of the material was dissolved in 0.1 M CH_3COONH_4 pH 8.0 and left to air oxidize for 10 days. The complete reduction or oxidization of the peptide was confirmed both by the Ellman reaction and by adding a 1000 molar excess of N-ethylmaleimide (NEM) to an aliquot of the peptide solution. In the later case, after incubation at 4 °C for 1 h, no increase of the polypetide MW was detected by mass spectrometry for the oxidized form while the reduced form presented a MW increase corresponding to the addition of four NEM molecules.

4.3 Immunization

Five- to six-weeks old female BALB/c mice were purchased from Harlan (Zeist, NL). Mice were injected subcutaneously at the base of the tail with 20 μg reduced or oxidized polypeptide dissolved in PBS and emulsified in IFA. For the short peptide PbCS 245–253, 4 μg were injected in combination with 100 μg of the universal helper peptide P30 [21]. Animals received a booster dose of immunogen after 3–4 weeks. Ten to twenty days after the boost, mice were bled to assess the production of specific antibodies. Subsequently, the animals were either sacrificed for proliferation, CTL or ELISPOT assays or exposed to sporozoite-bearing mosquitoes.

4.4 Parasite challenge

Plasmodium berghei (ANKA strain, clone 1, Dr. Walliker, Edinburgh or Dr. P.H. Lambert, WHO, Geneva) sporozoites were produced by cyclical transmission to laboratory-bred *Anopheles gambiae* or *A. stephensi* mosquitoes. Mice were anesthetized and exposed to infected mosquitoes. Mice were individually exposed to a previously determined number of bites necessary to obtain a complete infection in naive age-matched BALB/c mice. After parasite challenge, parasitemia was checked regularly from day 5 to day 14 by Giemsa-stained bloodsmears. Mice were considered protected when no parasites were detected 14 days after the challenge.

4.5 CTL induction by *in vitro* stimulation of spleen cells

Spleen cells (40–60 millions) were cultured in 10 ml of DMEM supplemented with 10 % FCS, 10 mM Hepes, 1% Glutamine and 50 μM β-mercaptoethanol; cells were cultured in 25 cm^2 tissue culture flasks (Falcon, Meylan, France) in the presence of 1 μM specific peptide. Cultures were harvested and tested for cytotoxicity 8–10 days after stimulation.

4.6 Cytolytic assay

P815 mastocytoma cells were labeled with sodium chromate (DuPont de Nemours) as described previously [18]. Labeled targets were co-incubated for 4 h with stimulated spleen cells in V-bottom 96-well plates (Greiner, Nürtingen,

Germany) at the indicated E:T ratios. Supernatants were then collected for chromium-release measurement. The percent specific lysis was calculated as: 100×(experimental-spontaneous release)/(total-spontaneous release).

4.7 Magnetic cell sorting

Cells from periaortic and inguinal lymph nodes and from the spleen were recovered from each individual mouse 10 days after the second peptide immunization. The isolated cells were washed two times with 10 ml of DMEM, 5% FCS and 1% Hepes. The spleen cells were further incubated at RT with 5 ml of ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.2–7.4) per spleen for 5 min, in order to lyse the red blood cells. The lysis was stopped by washing the spleen cells with 10 ml of DMEM, 5% FCS and 1% Hepes. Lymph nodes and spleen cells were re-suspended in PBS, 0.5% BSA and 2 mM EDTA and incubated at 4°C with anti-mouse CD8a (Ly-2) antibody conjugated to superparamagnetic MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min. The magnetic labeled cells were washed and applied onto the separation columns (Miltenyi Biotec). The negative selected cells were directly eluted from the columns and the labeled cells were flushed out after removing the column from the magnetic field. Cells were washed, counted and plated for the ELISPOT assay.

4.8 T cell depletion *in vivo*

Hybridomas H35 (CD8-specific rat IgG2b) [28] and GK1.5 (CD4-specific rat IgG2b) [29] were a gift from Katherina Hug (WHO-IRTC, Epalinges). Challenge was performed at day 0. One milligram of CD4-specific antibodies was injected at days –3, –2 and –1. Half milligram of CD8-specific antibodies was injected at days –2 and +2. Depletion was 95% during the time required for the complete development of *P. berghei* liver stages [30]. Depletion was verified by FACS analysis of PBL or spleen cells using CD4-specific FITC-labeled (Ref. 1300 024, Boehringer Mannheim, Germany) and CD8-specific PE-labeled (Ref. 1271 237, Boehringer Mannheim, Germany) antibodies.

4.9 ELISPOT assay

Nitrocellulose ELISPOT (Millipore, Molsheim, France) plates were coated overnight in a humid chamber at 4°C with a PBS solution containing 100 μg/ml of IFN-γ-specific antibody OIE703B2. Saturation step was performed by adding DMEM containing 10% FCS for 2 h at 37°C. Immune cells isolated from the draining lymph nodes of immunized mice were co-cultured for 24 h at 37°C in the plates with 100,000 irradiated P815 cells/well pulsed or not with the short peptide PbCS 245–253. Cells were then removed and a second IFN-γ-specific biotinylated antibody (ANI) was added (1 μg/ml in PBS-1% BSA) for 2 h at 37°C. After washing, streptavidin-alkaline phosphatase conjugate diluted in PBS-5% FCS was added for 1 h at 37°C and the presence of immune complexes revealed by the addition of BCIP/NBT substrate.

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