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RAPID COMMUNICATION

Augmentation of Human Influenza A Virus-Specific Cytotoxic T Lymphocyte Memory by Influenza Vaccine and Adjuvanted Carriers (ISCOMS)

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There is a need to improve the ability of subunit vaccines to induce $CD8^+$ CTL responses in humans, especially for vaccines used to prevent illness by organisms that undergo antigenic variation at their major neutralizing antibody sites, e.g., influenza A viruses and human immunodeficiency virus. Murine models have demonstrated the protective role of cross-reactive CTL against influenza A virus antigenic drift. We tested the ability of an adjuvanted carrier (Iscomatrix) to help human antigen-presenting cells present formalin-killed influenza vaccine to human $CD8^+$ CTL clones *in vitro* and in vaccinated humans. The results of a randomized, double-blind, controlled clinical study demonstrate that a single dose of a vaccine formulated into Iscom particles increased influenza A virus-specific CTL memory in 50–60% of recipients, compared to 5% of the recipients of the standard influenza vaccine. © 1999 Academic Press

Introduction. Influenza vaccines are composed of formalin-inactivated, detergent-disrupted virus, which is not efficient for MHC class I processing, but is designed to induce neutralizing antibodies to the major antibody combining sites on the hydrophilic end of the hemagglutinin (HA-1) subunit. Due to frequent mutations at these sites (*1*) the viral strains of influenza A (HI and H3) to be used in the vaccine need to be updated annually. Influenza virus-induced T cell responses, however, have much greater strain cross-reactivity (*2–5*), and we are interested in whether augmenting cross-reactive T cell memory might be an approach to improving influenza vaccines. There are convincing data from murine models that such cross-reactive $CD8^+$ CTL responses can be protective (e.g., *6–10*). We recognize that serotype-specific neutralizing antibody is the optimal approach to prevention of infection; nevertheless, in the presence of antigenic drift, cross-reactive memory $CD8^+$ CTL should restrict virus replication, reduce lung disease, and prevent deaths as they do in the mouse model. There is increasing evidence that in certain circumstances proteins and peptides can induce $CD8^+$ CTL responses *in vivo* and these responses can correlate with protection against challenge. Our laboratory reported that a fusion protein, which contained the influenza A (A/PR/8/34, H1N1) virus hemagglutinin HA2 subunit, induced $CD8^+$ CTL in mice, and this correlated with a significant reduction in pulmonary virus titers and protection against lethal challenge (*9, 10*). The pulmonary virus reduction and the survival from lethal challenge had the same specificity as the CTL responses. In mice immunized with this protein, no neutralizing antibodies were induced and the lung virus titers of all the H1 subtype virus strains tested (1934–1984) were significantly reduced despite antigenic changes at antibody combining sites (*10*). Lin and Askonas (*6*) had earlier reported the protective effect of a $CD8^+$ CTL NP-specific clone in mice challenged with influenza A virus, and we noted cross-reactive reduction in lung virus titers using a $CD8^+$ CTL clone specific for an epitope on the NS1 protein (*11*). Other studies demonstrated that osmotic shock (*12*) and the attachment of a fatty acid could help proteins and peptides induce $CD8^+$ T cell responses (*13*). Previous studies in humans with vaccines designed to induce or augment CTL responses to experimental vaccines, notably HIV-1 have yielded negative or disappointing results, with only a small minority of individuals responding even to pox virus-vectored genes (e.g., *14–16*). We wanted to determine whether the formulation of standard influenza vaccine into an Iscom carrier, which has been reported to increase murine antibody and CTL responses (*17, 18*), would augment CTL memory to influenza virus in humans. Iscoms are cage-like adjuvanted particles that have a diameter of approximately 30–40 nm, made up of glycosides (Quill A), cholesterol, phospholipids, and the

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TABLE 1A

Recognition by HLA B18 Restricted HA-Specific Human CD8$^+$ CTL Clone of A/Texas H1N1 Vaccine ± Iscomatrix Pulsed BLCL

HA-Specific clone	PR/8/34 Virus	A/Tx Virus	Iscomatrix[a]	A/TX Vaccine[b]	A/TX Flu-Iscom[c]
DS 10-2F5[d] (CD8$^+$)	53	33.8	−1.6	1.4	76.6
Min/Max	12.8	12.1	17.4	41.3	44.6

[a] Saponin concentration used was 337.5 μg/ml.
[b] HA concentration used was 82 μg/ml.
[c] HA concentration used was 57.5 μg/ml; saponin concentration was 337.5 μg/ml.
[d] E:T ratio = 10:1 to measure percentage of specific lysis.

protein or proteins of interest (*17*). A clinical study in which volunteers received under code (a) a standard dose of trivalent influenza vaccine containing 15 μg each of influenza A H1N1, H3N2, and influenza B virus subunits, (b) the standard vaccine formulated into ISCOM particles, or (c) a standard vaccine mixed with Iscomatrix but not formulated into particles. Clinical reactivity and antibody responses will be reported separately. The CTL results are reported in this article.

Results. Effects of Iscoms on specific recognition of target cells by human influenza A virus-specific CD8$^+$ CTL clones. Table 1A contains the results of a CTL assay with a CD8$^+$ HA-specific CTL clone and autologous B-LCL target cells exposed to a monovalent formalin-inactivated, detergent-disrupted A/Texas (H1N1) vaccine alone or after mixing with Iscomatrix. As expected there was no lysis of target cells exposed to the vaccine alone (1.4%), but there was a high level of specific lysis (76.6%) of target cells pulsed with the vaccine–Iscom formulation. Lysis of live virus-infected target cells was convincing as a positive control, and there was no lysis of target cells exposed only to Iscomatrix without a vaccine component, as a negative control.

We next tested the ability of a CD8$^+$ CTL clone specific for an HLA A2.1 restricted epitope on aa 122–130 of the influenza A virus NS1 protein to recognize autologous target cells exposed to recombinant NS1 protein. This clone is H1, H2, H3 subtype cross-reactive (*19*). The results in Table 1B demonstrate convincing killing of target cells pulsed with a peptide based on the sequence of aa 122–130 as the positive control and no lysis of target cells not treated with peptide as a negative control. There was no lysis of target cells exposed to either the NS1 protein or the Iscomatrix alone, but there was convincing lysis of target cells exposed to the mixture of NS1 protein and Iscomatrix. The lack of lysis of target cells pulsed with the NS1 protein without Iscoms was not due to a failure by the target cells to incorporate the NS1 protein, because a CD4$^+$ CTL clone specific for another epitope on the NS1 protein lysed NS1 protein-pulsed targets convincingly and to a similar degree when added alone or with Iscoms (data not presented).

Human CTL Responses to Vaccines. A clinical study was performed in 55 healthy adults (ages 18–45 years) and CTL assays were performed on PBMC obtained from samples of blood drawn on day 0 (prevaccination) and 14 and 56 days after vaccination with a single immunization with a formalin-inactivated vaccine ± Iscoms or Iscomatrix. We tested samples of PBMC obtained on days 0, 14, and 56 from each donor together in the same assay to eliminate variability of CTL assays performed on different days. In addition, we included as a positive control in each assay a well-characterized CTL clone to ensure that the virus-infected target cells were suitable for detecting specific lysis in the CTL assay performed that day. All assays were performed and were interpreted under code.

The data shown in Table 2 are examples that reflect the pattern of results observed in CTL assays performed with the PBMC of the 55 volunteers in the five vaccine groups. Donor 013 shows a modest level of influenza A virus-specific CTL memory on day 0 detected at multiple effector:target (E:T) ratios by the lysis of autologous targets infected with either the H1 or the H3 influenza A viruses that were in the vaccine. The levels of lysis by the

TABLE 1B

Recognition by Human HLA A2.1 Restricted NS1 aa 122–130-Specific CD8$^+$ CTL Clone of Recombinant NS1 Protein and Iscomatrix Pulsed BLCL

Clone	Uninfected	NS1 Peptide (aa 122–130)[a]	NS1 Protein[b]	NS1 Protein + Iscom[b,c]	Iscom[d]
DS 10-2C2[e] (CD8$^+$)	−6.2	88.4	−0.7	19.6	−11.0
Min/Max	17.8	19.6	17.3	29.9	21.3

[a] Peptide used at 25 μg/ml (killing observed down to 0.025 μg/ml also).
[b] Protein used at 35 μg/ml.
[c] Saponin used at 100 μg/ml.
[d] Saponin used at 70 μg/ml.
[e] E:T ratio = 10:1 to measure percentage of specific lysis.

TABLE 2

Bulk Culture Cytotoxic Activity by Effector Cells after Stimulation with the Homologous Virus of Infected Autologous Virus-Infected Target Cells[a]

	E/T	Donor 013 A/TX	Donor 013 A/JHN	Donor 050 A/TX	Donor 050 A/JHN	Donor 031 A/TX	Donor 031 A/JHN
Day 0	90	5.1%	11.3%	5.3%	17.2%	−0.4%	−1.1%
	30	4.6%	8.6%	1.7%	10.0%	−0.9%	1.1%
	10	5.2%	6.7%	0.3%	6.6%	0.7%	0.8%
Day 14	90	1.2%	15.3%	22.1%	36.7%	4.1%	14.1%
	30	3.1%	10.4%	15.1%	28.6%	2.0%	14.8%
	10	5.6%	6.5%	8.6%	17.5%	2.0%	6.3%
Day 56	90	3.7%	12.7%	22.5%	35.8%	7.1%	19.9%
	30	4.4%	12.7%	13.9%	25.2%	4.8%	12.0%
	10	4.2%	6.2%	7.1%	20.4%	2.3%	7.4%
Min/Max		14.6%	13.3%	17.7%	15.1%	16.2%	13.8%

[a] Values shown are (percentage lysis of virus-infected targets) − (% lysis of uninfected targets). Lysis values of uninfected targets were all <6%.

virus-stimulated PBMC obtained 14 and 56 days after vaccination were very similar to the prevaccination (day 0) level; therefore, this individual did not have any change in influenza A virus-specific CTL memory after vaccination. Most donors had these types of results. Donor 050 shows a different pattern. There is detectable influenza A virus-specific CTL memory on day 0 but it is increased significantly in the day 14 and 56 samples at multiple E:T ratios on both of the influenza A virus-infected target cells ($P < 0.01$). A third pattern of lysis is demonstrated by Donor 031. No detectable level of specific CTL memory activity was observed with the day 0 PBMC, but a modest and convincing level of CTL memory was detected on the postvaccination samples ($P < 0.01$) on days 14 and 56 at multiple E:T ratios and on target cells infected with the A/Texas (H1N1) or the A/Johannesburg (H3N2) viruses.

We interpreted the CTL data under code using a definition that an increase in the postvaccination sample of ≥10% SIL above the day 0 level in at least 2/3 E:T ratios was a ++ response, and an increase of ≥5% but less than 10% in SIL over the day 0 level was a + response. Increases in levels of lysis of ≥5% were uniformly found to be statistically significant. Increases in the percentage lysis of <5% were considered negative although some values were statistically significant. The CTL data were sent to the clinical monitor who organized the results into the vaccine groups. Table 3 summarizes the increases in influenza A virus CTL memory in the three vaccine groups. The CTL assay results showed a statistically significant ($P < 0.01$) increases in influenza A-specific CTL memory on days 14 or 56 (5.0–37.5% increases) in approximately 50% of the assays performed on PBMC of individuals who received one dose of vaccine formulated into Iscoms and in about 30% of the assays of individuals given vaccine mixed with the Iscomatrix, whereas only 5% of the CTL assays of the recipients of the standard vaccine had an increase in influenza A-specific CTL memory.

Discussion. The results presented in this paper demonstrate that it is possible to formulate protein antigens so that they can be processed and presented by HLA class I molecules and be recognized by human CD8$^+$ CTL clones *in vitro.* These results extend earlier reports that demonstrate murine CD8$^+$ cell recognition of epitopes on proteins that entered cells treated with osmotic shock or formulated into particles (*13, 14*). In these experiments we show that mixing with Iscomatrix enabled a formalin-killed monovalent influenza A virus vaccine to be recognized by a human hemagglutinin-specific CD8$^+$ CTL clone that is restricted by an HLA-B18. In addition, we found that an HLA 2.1 restricted epitope contained in a recombinant NS1 protein produced in *Escherichia coli* was also recognized on target cells by a human CD8$^+$ CTL clone after it was mixed with Iscomatrix; however, target B-LCL pulsed with the recombinant protein alone were not lysed.

These adjuvanted killed influenza vaccines also increased influenza A virus CTL memory activity in humans after a single dose. The memory CTL increases were seen in more recipients of the Iscom-formulated vaccines, with a smaller number responding to the mixture of vaccine and Iscomatrix. There was virtually no increase in CTL memory responses seen in the recipients of the standard vaccine alone. These increases in CTL activity correlate with increases in the number of influenza A virus CTL epitope peptide-specific IFNγ-producing cells detected using the PBMC of some of these

TABLE 3

CTL Responders[a] in the Flu-Iscom Phase 1 Trial

Virus	Flu-Iscom 50/45[b]	Flu-Iscom 75/45	Flu-Iscomatrix 50/45	Flu-Iscomatrix 75/45	Fluzone 45
H1 (A/TX/36/91)					
Nonresponders	4	6	10	7	11
5–10%	5	2	1	2	0
>10%	2	3	0	2	0
Responders	7	5	1	4	0
H3 (A/JHN/33/94)					
Nonresponders	7	4	7	6	10
5–10%	2	5	2	3	0
>10%	2	2	2	2	1
Responders	4	7	4	5	1

[a] Subjects showing from 5 to 37.5% increases in specific immune lysis in at least two E:T ratios by the day 14 or day 56 samples above the level of lysis by the day 0 samples.

[b] $\frac{\text{numerator = dose of iscomatrix}}{\text{denominator = dose of virus HA}}$.

vaccinees (Jameson *et al.,* unpublished data). These results demonstrate that quality-controlled human CTL assays can detect statistically significant increases in virus-specific CTL memory following administration of one dose of an inactivated vaccine. Furthermore, the findings demonstrate that formulating the vaccine as an Iscom, or even mixing the vaccine with Iscomatrix, results in augmented human CTL activity in vaccine recipients, which was consistent with the results of our preclinical *in vitro* human CTL assays. The results suggest that *in vitro* analysis with human T cell clones may be useful in predicting human immunogenicity and thus help to reduce the number of experimental constructs used in animal immunogenicity assays for the preclinical assessment of candidate vaccines.

It will be important to extend these types of studies of human CTL responses to other vaccines. The $CD8^+$ CTL responses to experimental HIV-1 vaccines have been very disappointing (*14–16*). A minority of vaccinees appear to develop CTL response even to pox-virus vectored vaccines, despite the use of multiple doses. The CTL data in the literature in response to HIV-1 vaccines are difficult to interpret. The PBMC of recipients of HIV-1 experimental vaccines were usually tested only after multiple doses often with dissimilar antigens, e.g., pox-vector priming and boosting with recombinant antigens, and fresh PBMC were tested in CTL assays; therefore pre- and postvaccine dosage samples of PBMC were not directly compared. Although cryopreservation is known to reduce the cytotoxic activity of activated T and NK cells, the PBMC of vaccine donors before and several weeks to months after receipt of an inactivated or subunit vaccine are not likely to contain activated cytotoxic T cells, but may contain vaccine-induced memory CTL. Another practical concern about the application of such quality-controlled CTL assays may be the work involved. Human CTL assays are complex, but the simultaneous testing of all samples from each donor in the same CTL assay removes assay-to-assay variation and the total number of CTL assays can be reduced compared to the CTL testing of individual samples of PBMC. Newer, more complex but quantitatively more sensitive assays are being developed to measure virus-specific CTL memory using as a read-out the number of peptide-specific $CD8^+$ T cells producing IFNγ after stimulation (*20*). We recently began to use such an assay and preliminary results (*19*) and Jameson *et al.* (unpublished data) support the observations reported here.

Materials and Methods. Volunteers. Healthy volunteers ages 18–45 years with no known allergy to eggs were recruited for the study. This was a randomized double-blind controlled study enrolling adults 18–45 years of age at a contract research organization. Prior to initiation, the protocol and consent were reviewed and approved by the institution's ethical review committee. Written informed consent was obtained from all participants prior to any study activity. Prospective participants were screened by medical history, physical examination, complete and differential blood cell count, an SMA-16 biochemistry panel, urine analysis, and drug testing. Prospective participants were excluded if they had received an influenza vaccination in the previous 6 months, were immunocompromised, were on immunosuppressive therapy, had a chronic illness requiring prescription medication, except for oral contraceptives, or had a suspected allergy to any component of the study vaccines. Female subjects were excluded if they were pregnant or lactating and were required either to be infertile or to be using an effective method of contraceptive for the duration of the study. A urine pregnancy test was conducted on all female participants just prior to vaccination. At the time of immunization, subjects were required to be free of any signs or symptoms of respiratory illnesses such as coughs or congestion. Fifty-five healthy adults were enrolled in the study. Eleven subjects were enrolled into each of the five study groups: Fluzone (the licensed standard vaccine), 75 μg Flu-Iscoms, 50 μg Flu-Iscoms, 75 μ Flu-Iscomatrix, and 50 μg Flu-Iscomatrix. Study vaccine was administered by intramuscular injection into the right or left deltoid muscle using a needle not less than 1 in. in length. All subjects were contacted daily by telephone for 7 days after vaccination to monitor local and systemic reactions. Subjects were also monitored for 56 days after vaccination for adverse events.

Blood samples were taken for T cell studies on days 0 (prevaccination) and on days 14 and 56 after vaccination. Blood samples taken on days 0, 28, and 56 were tested for antibody responses. Serologic and safety results will be presented in another publication. All subjects were vaccinated and returned for follow-up on the same day. All 55 (100%) subjects completed the required follow-up visits according to protocols.

Influenza Vaccines. The influenza antigens used to prepare the influenza Iscom vaccines were the monovalent subvirion concentrate pools of the 1994/1995 vaccine strains, A/Texas/36/91/X-113 A/Johannesburg/33/94/RESVIR-8 and B/Harbin/7/94. These materials were manufactured under GMP at Connaught Laboratories Inc., Swiftwater, Pennsylvania. The different formulations were studied to help learn which formulations would be better for antigen presentation and to determine whether 75 or 50 μg of Iscomatrix was more active. There were five study groups each with 11 volunteers who received the standard vaccine alone, vaccine in Iscoms at 75 or 50 μg, or vaccine mixed with Iscomatrix at 75 or 50 μg/dose. All clinical and laboratory studies were performed under code.

Production of Influenza Iscom Trivalent Vaccines. Flu-Iscom vaccines were prepared as described previously (*18*) with some modifications. Briefly, monovalent sub-

virion vaccine at 0.1–0.4 mg/ml in PBS (10 mM, pH 6.8, containing 0.5% v/v 2-phenoxyethanol) was mixed with phosphatidyl choline and cholesterol (each at 0.2–0.8 mg/ml in 20% Mega-10) and 1–4 mg/ml Iscoprep 703 (Iscotec, Lulea, Sweden) for 2 h at 25°C. Removal of Mega-10 and concentration of formed Flu-Iscoms were achieved by diafiltration (with PBS) and ultrafiltration using a 30-kDa cut-off membrane (Applikon). Trivalent Flu-Iscom vaccines were prepared by combining individual monovalent preparations to give the desired proportion of HA and Iscoprep. Iscomatrix particles were prepared as described above for Flu-Iscom monovalent vaccines except the Iscom particles were prepared in the absence of monovalent subvirion vaccine. The trivalent Flu-Iscomatrix was prepared by combining Iscomatrix with a mixture of the three monovalent vaccines to give the desired proportion of HA and Iscoprep. Formation of Iscom particles was verified by negative-staining electron microscopy and the HA content by single radial immunodiffusion (SRID) using antigen and antiserum reference standards (CBER, Bethesda, MD). The Iscoprep content was determined on samples extracted with chloroform–methanol and dried. Samples were reconstituted in 30% acetonitrile and run on a C4 reverse-phase HPLC column using a gradient of 30–40% acetonitrile containing 0.15% trifluoroacetic acid. The final formulations of trivalent Flu-Iscoms and Flu-Iscomatrix comprised approximately 45 μg HA (15 μg per strain) and either 50 or 75 μg of Iscoprep per 0.5-ml dose. Trivalent vaccine was formulated as 45 μg HA (15 μg per strain) per 0.5-ml dose. All vaccines passed release criteria established for sterility, endotoxin activity, and potency.

Collection and Preparation of Blood for CTL Studies. Approximately 60 ml of venous blood was obtained from each volunteer just prior to vaccination (day 0) and 14 and 56 days after vaccination using the Vacutainer CPT Tubes (Becton Dickinson, Franklin Lakes, NJ) with sodium citrate. Tubes were centrifuged within 2 h of blood collection. Processing of the blood sample to yield PBMC were performed as recommended by the manufacturer. To collect the cells, the entire contents of the tube above the gel were pipetted into a separate 50-ml centrifuge tube, 1× PBS was added, the cells were mixed by inverting the tube five times and centrifuged for 15 min at 300 RCF. Supernatants were aspirated and the cell pellet was resuspended by gently vortexing, 1× PBS was added, and cells were mixed again by inverting the tube five times followed by centrifuging for 10 min at 200 RCF. Supernatant was again aspirated and the PBMC were resuspended at a concentration of 1.0×10^7/ml in RPMI–20% heat-inactivated fetal bovine serum (HIFBS) + 10% DMSO and cryopreserved in liquid nitrogen using a programmable freezer.

CTL Assays. Preparation of effector cells. A vial of cells from the day 0 bleed of each donor was thawed to be used as stimulator cells. The cells were washed with RPMI–10% serum HIFBS and 5×10^6 cells were added to each of two polypropylene tubes and centrifuged. Then 0.1 ml (approx. 1×10^8 PFU) of either A/TX or A/JHN virus was added to 0.4 ml of PBS + 0.1% bovine serum albumin (Gibco Life Technologies) per tube and incubated for 90 min at 37°C. Cells obtained from each donor on days, 0, 14, and 56 were thawed and 5×10^6 cells were added to each flask in RPMI–10% HIFBS medium to be used as responder cells. The stimulator cells were washed twice and 5×10^5 cells were then added to each flask of responder cells using three flasks of cells for each virus stimulation. Flasks were incubated for 6 days at 37°C in 5% CO_2 and 95% humidity. Recently reported CTL clones (*19*) were used as a positive control in each assay.

Target Cells and CTL Assays. EBV-transformed B-LCL were prepared as described previously (*19*). Five days after the bulk culture effector cells were established, 3×10^5 BLCL from each donor were infected with 0.1 ml of each influenza A virus diluted in 0.4 ml of RPMI–10% FBS (an m.o.i. of about 30:1) in a 48-well plate and incubated for 14–18 h. The next day, the target cells were washed once and labeled with ^{51}Cr for 60 min. The effector cells were washed and were added to achieve 90, 30, and 10 effector:target ratios. Then 1.5×10^4 unlabeled K562 cells/well were added to each well to decrease NK cell activity against target cells. The target cells were washed, 1.5×10^3 cells were added to each well in triplicate, and plates were spun at 1000 RPM for 5 min. The plates were incubated for 5 h at 37°C in 5% CO_2 and were harvested using the Skatron Supernatant Collection System. The percentage specific immune lysis was calculated by

$$\frac{\text{mean CPM Exp} - \text{mean CPM Min}}{\text{mean CPM Max (Renex)} - \text{mean CPM Min}} \times 100 = \text{percentage specific immune lysis.}$$

The Min/Max ratio is the percentage of the maximum release of ^{51}Cr by detergent lysis of target cells (Max) by the medium control wells (Min). Statistical analysis was performed using the Student *t* test to compare the means of three replicate samples at each E:T ratio of the postvaccination to the prevaccination stimulated PBMC.

In Vitro Analysis of Target Cell Lysis by Human CD8+ T Cell Clones after Exposure to Influenza Vaccine and Recombinant Proteins ± Iscomatrix. We tested the ability of human CD8+ CTL clones to recognize autologous B-LCL following exposure to monovalent formalin-inactivated detergent disrupted A/Texas (H1N1) vaccine virus alone and after formulation into Iscom particles using an HLA-B18 restricted CD8+ CTL clone specific to the H1 hemagglutinin, which has been recently described (*19*). Vaccine alone containing 230 HA units or vaccine formulated into Iscoms containing 230 HA units was added to

B-LCL target cells and incubated at 37°C for 1 h, washed, and reincubated at 37°C for 24 h followed by ^{51}Cr labeling for use as target cells in a CTL assay with the CTL clone. In addition we tested another $CD8^+$ CTL clone, which is HLA-2.1 restricted and recognizes an H1-H2-H3 cross-reactive CTL epitope on NS1 aa 122–130, for recognition of this epitope on recombinant NS1 protein expressed in *E. coli.* The recombinant NS1 protein and Iscomatrix were mixed 24 h earlier and incubated at 4°C before addition to target cells. The final concentration of NS1 protein was 35 μg/ml and the final concentration of saponin was 100 μg/ml. After 24 h at 4°C, either the control recombinant protein or the mixture of recombinant protein and Iscomatrix was added to B-LCL target cells, which were washed after 1 h, incubated for 24 h at 37°C, and ^{51}Cr labeled. The CTL assay was performed as above.

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